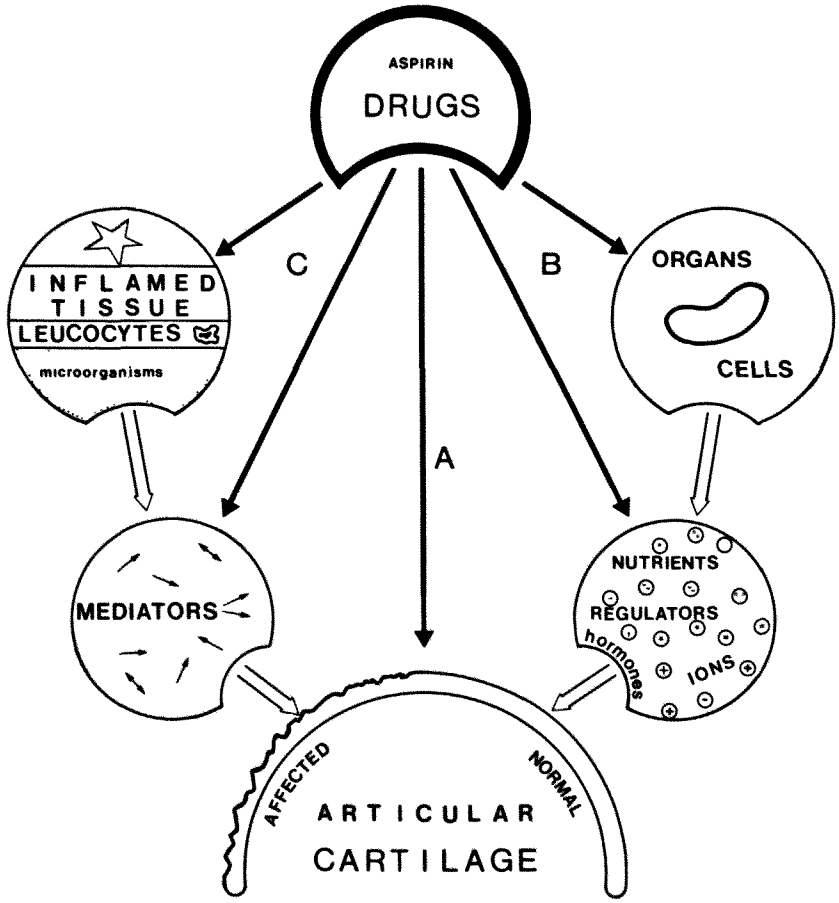
MURINE PATELLAR CARTILAGE AND ITS SUSCEPTIBILITY TO NON STEROIDAL ANTIINFLAMMATORY DRUGS



Bernard J. de Vries

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TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR B.M.F. VAN IERSEL VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 20 FEBRUARI 1987 DES NAMIDDAGS TE 1.30 UUR PRECIES.

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Aan Jessy, Kim en Kyra
Aan mijn moeder

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CHAPTER 1

VARIATIONS IN THE SUSCEPTIBILITY OF ARTICULAR CARTILAGE TO ANTIRHEUMATIC DRUGS

Bernard J de Vries, Wim B van den Berg and Levinus BA van de Putte

VARIATIONS IN THE SUSCEPTIBILITY OF ARTICULAR CARTILAGE TO ANTIRHEUMATIC DRUGS

1.1 INTRODUCTION

Joint diseases, especially the chronic ones, are often associated with pathology of the articular cartilage. Basically there are two types of joint disease: a. Inflammation of the synovial tissue, which secundarily leads to pathological changes of the cartilage; the prototype of this condition is rheumatoid arthritis (RA). b. Primary pathology of the articular cartilage, which subsequently can give rise to (mild) inflammatory changes in the synovial tissue. The prototype of this condition is osteoarthrosis, also called osteoarthritis (OA). So, in (chronic) joint diseases we often find the combination of synovial tissue inflammation and cartilage pathology. Clinical symptoms and signs of this conditions include pain, stiffness, limited mobility and signs of inflammation. Therapy is directed at two goals: 1. suppression of inflammation, and 2. relief of pain and stiffness.

As far as drug treatment is concerned the clinician has a vast armamentarium of antirheumatic drugs (ARDs) to be used for these purposes. First, there is the group of nonsteroidal antiinflammatory drugs (NSAIDs). These drugs are used in both OA and RA. They suppress both pain and inflammation, act quickly but only for a short period of time, and generally do not alter the disease course in a positive way. In addition there are other drugs, mainly used in the treatment of RA, including the so called slow acting drugs (antimalarial drugs, gold compounds and D-penicillamine), cytotoxic drugs (azathioprine, methotrexate) and corticosteroids. Except for the corticosteroids, these drugs only become effective several months after institution of therapy and do not have a direct effect on pain and stiffness. Apart from their effect on the inflammatory process, ARDs are not believed to have any beneficial effect per se on pathological cartilage, except for some occasional reports on salicylate (18, 25, 27). In contrast, experimental investigations (see below) and an occasional clinical study (71) suggest that ARDs may be potentially detrimental to articular cartilage.

The present investigation was undertaken to study the, possibly negative, effects of NSAIDs on hyaline articular cartilage, using the mouse as the experimental animal. Before presenting the aim of this study, we should like to evaluate what is known at present on the iatrogenic effects of ARDs on cartilage proteoglycan metabolism, and, furthermore, on the test systems employed and their pitfalls.

1.2 IATROGENIC EFFECTS ON CARTILAGE PROTEOGLYCAN METABOLISM

Experimental studies on normal cartilage. In pathologic cartilage the metabolism of proteoglycans (PGs), besides water and collagen the major constituents of cartilage, is first affected, at least in the early phase of most joint disorders. Therefore, many drug studies on cartilage cell (chondrocyte) function were and are directed on PG metabolism; little information is available on modulation of collagen metabolism by drugs (103). By far the greatest part of cartilage PGs consists of sulfated glycosaminoglycans (GAGs) of which the turnover can easily be measured with radiolabeled precursors. Frequently, radiosulfate incorporation is used as an indirect measure for PG formation or loss.

As shown in Tables 1 and 2 variable effects have been reported for one particular drug and this holds for a large number of ARDs of va-

Table 2. Effect of antirheumatic drugs on the basal rate of glycosaminoglycan degradation by normal cartilage cells in vitro.

Antirheumatic drug	GAG degradation in vitro ↑	—	↓	(Refs)
Hydrocortisone		•	•	(20,113)
Sodium salicylate		•	•	(20,108,113)
Phenylbutazone		•	•	(20,113)
Benoxaprofen		•		(11)
Ibuprofen		•		(74)
Fenoprofen			•	(74)
Tiaprofenic acid		•		(72)
Indomethacine			•	(20)
Sulindac sulfide		•		(74)
Piroxicam		•		(38)
D-penicillamine		•		(20)
Chloroquine		•		(113)
Colchicine			•	(20)
Arteparon			•	(1)

↑ increase, ↓ decrease, —no change.

Table 1. In vitro and in vivo effects of antirheumatic drugs on the basal rate of glycosaminoglycan synthesis by normal cartilage cells.

Antirheumatic drug	GAG synthesis In vitro ↑	—	↓	(Refs)	In vivo ↑	—	↓	(Refs)
SAIDS*								
Cortisone			•	(112,113)			•	(2)
Hydrocortisone (cortisol)	•	•	•	(21,51,64,82,112)		•	•	(10,37)
Hydrocortisone-acetate					•	•	•	(37)
Prednisone		•		(113)				
Prednisolone			•	(54,113)			•	(54)
Prednisolone-acetate						•	•	(37)
Triamcinolone			•	(113)				
Dexamethasone	•		•	(82,85,113)	•	•	•	(37)
NSAIDS*								
Salicylates:								
Acetylsalicylic acid (aspirin)		•	•	(21,112,113)	•	•		(22,37)
Salicylic acid/salicylate	•	•	•	(64,74,108,112,114)		•	•	(10,16,107)
Salicylamide		•	•	(112,113,114)			•	(34)
Methylsalicylates			•	(114)				
Pyrazoles								
Phenylbutazone			•	(113,114)			•	(10,54)
Oxyphenbutazone			•	(113)		•	•	(10,37)
Phenylacetic acid derivative:								
Diclofenac		•		(70)		•		(24)
Propionic acid derivatives:								
Benoxaprofen	•			(11)				
Ibuprofen		•	•	(64,74)		•		(16)
Naproxen			•	(106)				
Flurbiprofen		•		(64)				
Fenoprofen			•	(74)				
Tiaprofenic acid	•	•		(72)			•	(72)
Indoles								
Indomethacin	•	•	•	(21,52,64,74,115)		•	•	(24,111)
Sulindac sulfoxide		•	•	(74,106)		•		(16)
Sulindac sulfide	•	•		(70,74)				
Tolmetin			•	(11)				
Fenamates								
Metenamic acid			•	(45)				
Flufenamic acid							•	(10)
Oxicams								
Piroxicam		•		(38,70)				
Isoxicam			•	(11)				
Others:								
Diftalone							•	(14)
Paracetamol		•		(113)				
GOLD COMPOUNDS								
Gold sodium thiosulphate			•	(115)				
Aurothioglucose	•	•		(70,110)	•			(110)
Auranofin		•		(70)				
Aurothiomalate		•	•	(113)				
ANTI MALARIALS								
Chloroquine			•	(113)			•	(115)
Hydroxychloroquine			•	(113)			•	(115)
Chloroquine diphosphate			•	(113)		•		(10,115)
OTHERS								
Colchicine			•	(3)				
Calcitonin	•	•	•	(63,101)				
Arteparon	•		•	(1,47,105)				

* (N)SAIDS = (non)steroidal anti-inflammatory drugs. ↑ increase; ↓ decrease; — no change.

rious groups and chemical classes (see the nonsteroidal antiinflammatory drugs = NSAIDs). The drug effects summarized were examined on the GAG metabolism of normal cartilage and, in some cases of in vitro studies, also on isolated cartilage cells (chondrocytes) following either short-term in vivo or in vitro use. The variability is presumably a consequence of the use of different biochemical assays, the source of cartilage, or because of biological variability, existing in analogous specimens derived from different animal species (see below). Also very important, in this respect, is the concentration or dosis of drug used in the various studies.

For example, it appears that in vitro very low concentrations of some drugs, corresponding roughly with their free (i.e. non-protein bound) therapeutic concentrations, stimulate basal GAG synthesis. No effect or even the reverse is seen if higher concentrations are used (hydrocortisone, dexamethasone, salicylic acid, indomethacin). Unfortunately, only a few drugs are examined at these low concentrations, so at present it is not known if more ARDs exist with potentially stimulating properties. In addition, there are also reports on drugs devoid of any suppressive effect, some of which even stimulate GAG synthesis. This holds for broad concentration ranges up to very high (nontherapeutic) concentrations (diclofenac, benoxaprofen, tiaprofenic acid, sulindac sulfide, piroxicam, aurothioglucose). These latter drugs would potentially deserve first choice for therapeutic use if their in vivo effects, concerning cartilage, were identical with those in vitro. However, this choice strongly depends also on other (potential) harmful side effects. Due to detrimental side effects, the promising drug benoxaprofen was withdrawn from the market shortly after its introduction in 1982.

Data on in vivo drug effects on normal cartilage are more scanty and the results, as far as the data in Table 1 are concerned, do not always correspond with in vitro data. Like in vitro, also in vivo low doses of steroids tend to stimulate cartilage GAG synthesis (37), an effect not seen at higher doses (hydrocortisone-acetate and dexamethasone).

It is striking that, as far as we know, no data exist on potential drug effects on in vivo basal cartilage GAG degradation, except those of de Vries et al (107). Only eight references were found on in vitro effects of drugs on degradation (Table 2). If both basal synthesis and

degradation are disturbed to the same extent by a drug, there would be no change in the integrity of cartilage, unless PG properties would be affected. Consequently, the data in Table 1, on their own, do not inform about any net effect on cartilage!

It is of interest that no drug exerts an accelerating effect on the basal GAG degradation rate (Table 2). Some of these studies were performed concomitantly with synthesis studies (Table 1) under almost identical conditions. These data are summarized in Table 3. Tentative conclusions can be made about a net effect of these drugs on basal cartilage PG content (increase/decrease). With the available in vitro data (Table 3), one would anticipate that tiaprofenic acid, piroxicam, sodium salicylate and perhaps fenoprofen are harmless to normal cartilage in vivo. However, sodium salicylate (107) was found to significantly suppress in vivo GAG synthesis in articular cartilage of healthy mice, without an effect on in vivo GAG degradation. Theoretically this would lead to PG depletion if healthy mice were treated chronically with this drug. Nevertheless, light microscopical observations revealed no changes in articular cartilage (stained for PG) after daily treatment for 7.5 weeks (72, Chapter 6 and 8). In this particular case the results of

Table 3. Net effect of antirheumatic drugs on the basal glycosaminoglycan metabolism by normal cartilage cells in vitro.

Antirheumatic drug	GAG metabolism		Tentative conclusion: net effect	(Refs)
	synthesis	degradation		
Benoxaprofen	↑	—	PG-increase	(11)
Sulindac sulfide	↑	—	PG-increase	(74)
Ibuprofen	↓	—	PG-depletion	(74)
Fenoprofen	↓	↓	No effect?	(74)
Tiaprofenic acid	—	—	No effect	(72)
Piroxicam	—	—	No effect	(38)
Chloroquine	↓	—	PG-depletion	(113)
Sodium salicylate	—	—	No effect	(72,108)

↑ increase, ↓ decrease, — no change

short-term in vivo studies (inhibition of cartilage GAG synthesis) are not predictive for gross changes in cartilage after long-term drug treatment. Slight changes in proteoglycan quality cannot be excluded yet and are currently under investigation. No explanation for this discrepancy is at hand, perhaps it has something to do with the reversibility of the described drug effects (72), another aspect often neglected in chondrocyte metabolic studies. Perhaps chondrocytes may also show adaptation upon long term drug treatment.

Experimental studies on pathological cartilage. On the basis of the contradictory observations, no generalization can be made as to the potential role of ARDs in disturbing basal cartilage GAG metabolism. Furthermore, the experiments were performed on healthy cartilage, frequently not of articular origin. The practice of medication with ARDs comprises human subjects with already more or less deteriorated articular cartilage. So it is more rational to investigate potential side effects of drugs under these conditions. Except for the group of Palmoski and Brandt (78) who observed an aggravated suppressive effect of salicylate and indomethacin on the augmented PG synthesis of OA cartilage, no in vitro drug studies are done with (osteo)arhritic cartilage. In vivo some efforts have been made in experimental models of OA. However, in contrast to the findings in vitro, the altered in vivo PG synthesis in OA cartilage was not influenced by the administration of salicylate, ibuprofen, sulindac sulfoxide, diclofenac or indomethacin (16,23,24). Apart from our study (Chapter 7) no reports exist about in vivo drug effects on the adversely affected PG metabolism in articular cartilage of animals with experimentally induced inflammatory arthritis. Long-term drug studies with experimental animal models of OA and RA are of utmost relevance, since they resemble medical practice. Studies on this issue have been performed already for several years, however, again with conflicting results as far as the integrity of articular cartilage is concerned (Table 4). In experimental animal models of inflammatory arthritis, the attention, hitherto, was more directed to the antiinflammatory properties of ARDs than on potential chondrotropic properties. Except perhaps for glucocorticoids and immunosuppressives or disease modifiers like azathioprine and sulphasalazine, there are no clear

indications of any NSAID induced attennuation of cartilage erosions, caused by experimental joint inflammation (40-42).

With all these data in mind we realize that it remains arduous to the physician to make conclusions about potential detrimental or beneficial effects of particular ARDs towards human articular cartilage. These drug effects might be entirely determined by the kind of experimental test system in use. The relevance and value of some test systems are doubtful but will not be evaluated apart; instead a general discussion about this subject will follow. It will be confined particularly to the NSAIDs, drugs commonly used in antirheumatic therapy, and variables present in cartilage test-systems, both in vitro and in vivo, which might influence chondrocyte metabolic activity and perhaps drug susceptibility and which may lead to false conclusions. Owing to their potential predictive value and relative inexpensiveness, short-term studies are very important for the clinician as well as for the pharmaceutical industry, designing and developing new ARDs. Nevertheless, promising drugs should always be tested in vivo and preferably in arthritis and arthrosis models.

Table 4. Effect of long-term treatment (several weeks) with some NSAIDs on degenerative articular cartilage of experimental animal models.

Antirheumatic drug	Severity of cartilage erosion			References
	increased	unchanged	reduced	
Salicylate	•	•	•	(11,19,25,28,28[a],96,116)
Indomethacin	•	•	•	(19,23,116)
Phenylbutazone	•	•		(19,116)
Ibuprofen	•	•		(19,116)
Piroxicam		•	•	(19,61)

1.3 CARTILAGE TEST SYSTEMS: AN EVALUATION.

1.3.1 CARTILAGE

Cartilage is a tissue lacking most of the attributes of other body tissues: it has no nerves, no blood vessels and no lymphatic system. It is a tissue whose properties are established not by the properties of its cells (chondrocytes), but by what the cells secrete: an elaborate network of giant molecules the cells deposit around themselves to form an extracellular matrix. The matrix includes some of the largest proteins made by any cell in nature: proteoglycans. It also includes collagen and great volumes of water. These three major constituents determine the cartilage properties: tensile strength and resilience (17).

Type and distribution of cartilage (5,17). There are three types of cartilage: fibrocartilage, elastic- and hyaline cartilage. The molecular structure of each type of cartilage governs its properties, needed for specialized functional requirements. They will briefly be discussed here with reference to Figure 1.

Fibrocartilage (f) is found among others in the intervertebral disks and the interarticular disks of the knee joints (menisci). It also blends in with tendons and ligaments, particularly at their junction with bones. Compared to hyaline cartilage, it contains more collagen and less proteoglycans leading to higher tensile strength.

Elastic cartilage (e) is found where more elasticity is required and predominates for example the cartilage of the auricle of the ear and the front of the rib cage; it contains a normal amount of collagenic fibers but has, in addition, elastic fibers.

Hyaline cartilage (h) occurs in nearly all the fetal skeleton. Later, in the growing body, the epiphyseal plates at the tips of bones are still composed of this type of cartilage. Throughout life, hyaline cartilage provides a covering for the ends of the bones that withstands compressive forces yet enables the bones to slip over each other smoothly. It is also found in the nose, larynx, trachea and bronchi. With the exception of the free surfaces of articular cartilage, hyaline cartilage is always invested by a perichondrium.

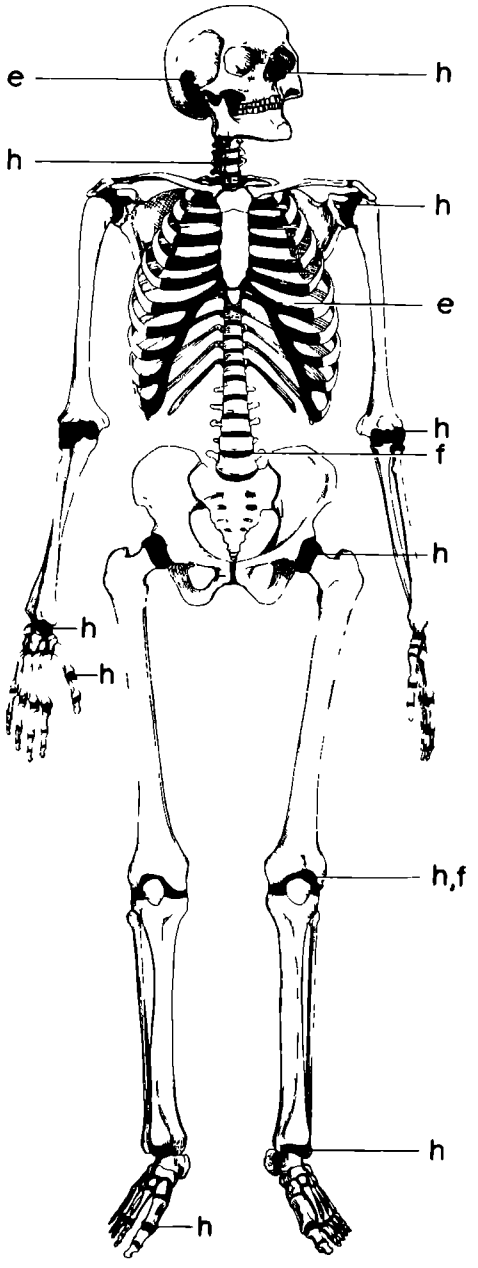

Figure 1. Some locations of three different types of cartilage in the human skeleton. f = fibrocartilage; e = elastic cartilage; h = hyaline cartilage.

Cartilage source for drug studies. Several kinds of cartilage, often derived from different species, were used to study ARD effects on cartilage metabolism and properties (section 1.2). Regarding the age at which joint diseases occur, young and adult articular cartilage are most relevant for these studies. Experimental drug studies on fetal cartilage might serve to elucidate potential teratogenic effects, due to iatrogenic harm in pregnant rheumatoid patients (34,92). The composition and metabolism of articular cartilage will now be reviewed shortly.

Articular cartilage proteoglycan. The major molecular components of articular cartilage - the molecules known to be responsible for the tensile strength and resilience of the tissue - are collagen and proteoglycan. The collagen of articular cartilage differs from collagen of other interstitial connective tissues and is termed, type II. Because collagen metabolism is almost inert in normal adult cartilage, and considered not to be involved in early stages of joint disorders, this component will not be discussed here; see for review Lane and Weiss (56).

Proteoglycan monomers (PGs) are constructed of a protein core to which large numbers of highly sulfated glycosaminoglycans (GAGs), termed chondroitin sulfate (CS) and keratan sulfate (KS), are attached. These GAGs have variable chain lengths and, in addition with several types of short length oligosaccharides, are covalently bound to the protein core in a lateral way (Figure 2A). CS consists of repeating units of glucuronic acid and N-acetylgalactosamine. In the average chain of CS (MW 20,000) there are about 25-30 such disaccharide units, and there is almost one sulfate group per disaccharide. KS is more variable than CS in both chain length (MW 5000-10,000) and in degree of sulfation. It does not contain uronic acid and is essentially build up of repeating disaccharide units (about 13) of N-acetylglucosamine and galactose. The current model for cartilage PG has a central core protein to which about 100 CS and 50 KS chains are attached to yield an overall molecular weight of $1\text{-}4\times10^6$ daltons (Figure 2A). Bound to hyaluronic acid (HA), another, non-sulfated GAG (MW 500,000), comprising 1-5% of the cartilage content, very large aggregates are formed with

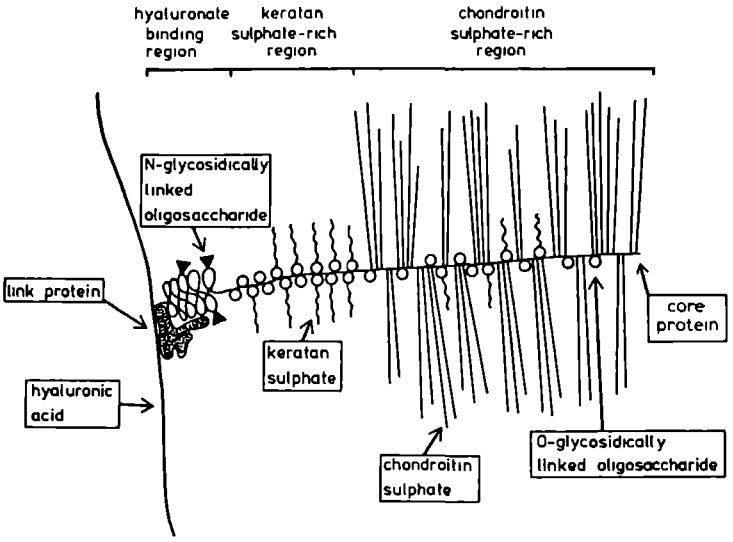

Figure 2A. Model of a proteoglycan monomer bound to hyaluronic acid.

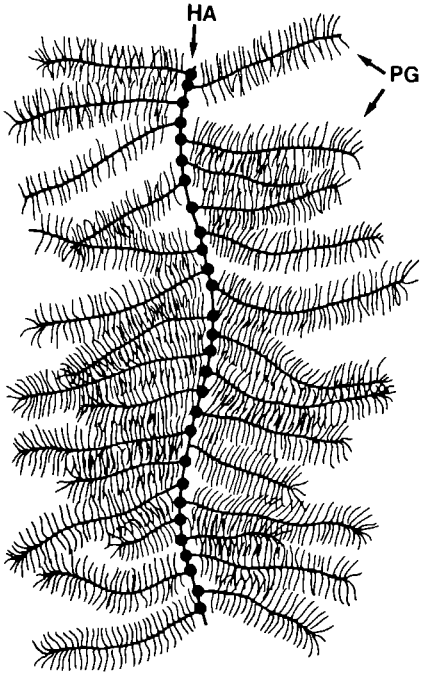

Figure 2B. Model of a proteoglycan aggregate. PG = proteoglycan monomer; HA = hyaluronic acid.

molecular weights extending to 10^8 daltons (67). This interaction is stabilized by the additional, non-covalent, binding of link proteins to the PG and to HA (Figure 2B).

PG-GAG synthesis: radioprecursor incorporation. The sulfated GAGs, CS and KS, are not formed if core protein synthesis is ceased. The core protein, shortly after its synthesis or during intracellular transport, serves as a primer on which GAG chain synthesis is initiated. An additional key step in the synthesis of PG-GAGs is the metabolic activation of inorganic sulfate prior to its incorporation into the growing carbohydrate chain. Sulfate conjugation requires 3-posphoadenosine-5-phosphosulfate (PAPS) as an intermediate. The synthesis of PAPS proceeds via two steps: first, activation of sulfate to form adenosyl phosphosulfate (APS), catalyzed by ATP-sulfurylase, followed by phosphorylation to form PAPS, catalyzed by APS-kinase:

$$1.\quad SO_4 + ATP \longrightarrow APS + PP_i$$

$$2.\quad APS + ATP \longrightarrow PAPS + ADP$$

(ATP=adenosine triphosphate;AD=adenosine diphosphate;PPi=pyrophosphate) Recent studies with brachymorphic mice suggest that the availability of the sulfate donor PAPS may be the rate-limiting step in the sulfation process. Without disturbing GAG chain elongation, decreased sulfation can be achieved by a defect in ATP-sulfurylase or APS-kinase (100) as well as by a decreased supply or production of SO_4 (69) or ATP (45, 114); these latter events are induced by some antiinflammatory drugs (30,92,103,107, and Chapter 4). Since the sulfated GAGs are practically the only molecules in articular cartilage to which sulfate is attached (66) and sulfation proceeds concomitantly with GAG chain elongation, radiosulfate is an useful radioprecursor to study ultimate PG turnover in cartilage (see also Chapter 2). Of course, other radiolabeled precursors can be used to study GAG chain metabolism (e.g. ^{3}H-glucosamine, ^{3}H-glucose, ^{3}H-acetate), as shown for CS and HA in Figure 3, but they are less specific for GAGs in that they also incorporate in other molecules present in cartilage (e.g. glycoproteins). Moreover, in contrast

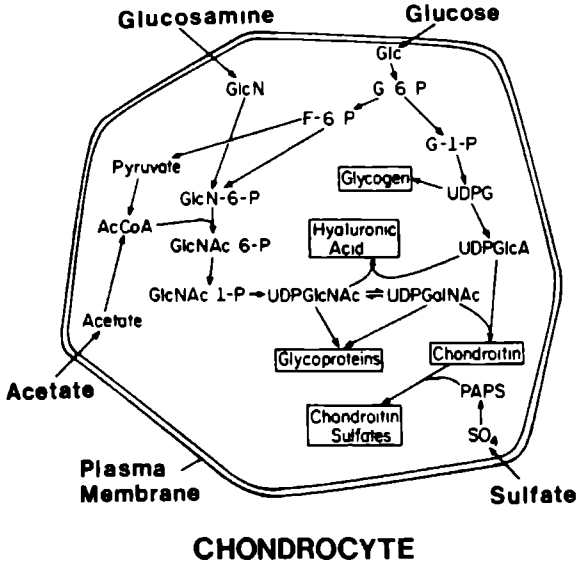

Figure 3. Precursors to assess chondrocyte GAG metabolism.

to $^{35}SO_4$, they have to proceed through a large intracellular hexosamine pool before they become incorporated into growing GAG chains, making their use for quantitative measurements less attractive (109).

Pathological cartilage. In most joint disorders PG metabolism and properties are adversely affected whereas, at least in the early stages of the diseases, collagen remains relative intact.

Osteoarthrosis or osteoarthritis (OA) is a disease of joints that leads to progressive deterioration of the articular cartilage. The focal nature of the characteristic OA lesion and its localization in individual joints suggest that mechanical destruction of the articular cartilage is an essential pathological process in the development of OA, however systemic factors cannot be excluded (43). Several investigators have detected a decrease in PG content and an increase in PG synthesis in OA articular cartilage (58,90). The chondrocytes apparently attempt to replenish the PG depletion. Since the rate of degradation

of the PGs in OA cartilage exceeds their rate of synthesis, drugs which stimulate synthesis or retard degradation would perhaps be of benefit.

Rheumatoid arthritis (RA), is a systemic disease with unknown etiology. It is accompanied by severe destruction of articular cartilage. Studies made on carrageenin-, zymosan- or antigen-induced inflammatory arthritis in experimental animals (9,55,60), models exhibiting features of RA, demonstrated not only an accelerated breakdown of cartilage PGs but also a markedly reduced PG synthesis. These processes ultimately result in severe depletion of matrix PGs and might even lead to chondrocyte death (55). Probably, because suppressive factors remain present in the joint fluid, chondrocytes are not able to restore the PG depleted matrix.

Since the biochemical response of joint tissues is markedly different in RA and OA, drugs developed to treat the former are unlikely to be of benefit in the latter.

1.3.2. EFFECTS OF DRUGS ON ARTICULAR CARTILAGE

Drugs can exert their effects on cartilage in three ways (Figure 4):

A. A direct effect by interfering with the chondrocyte metabolic machinery i.e. disturbance of degradative and synthetic processes of proteins, proteoglycans (PG) and glycosaminoglycans (GAG). Enzymes involved in the degradative processes are probably present both within the chondrocyte and within the surrounding matrix and drug effects are expected at both sites. Direct drug effects can only be studied in vitro. In vivo, drugs may influence systemic factors, important for cartilage turnover (see below), making proper interpretation of mechanistic actions nearly impossible.

B. An indirect effect by influencing (systemic) essential endogenous factors necessary for growth and development, or for maintaining optimal function of cartilage. For instance, supply of nutrients in its most broad sense (large molecules like proteins, carbohydrates, amino acids and small molecules like inorganic ions) and growth factors (vitamins, somatomedins) come into consideration. These studies can only be performed in vivo.

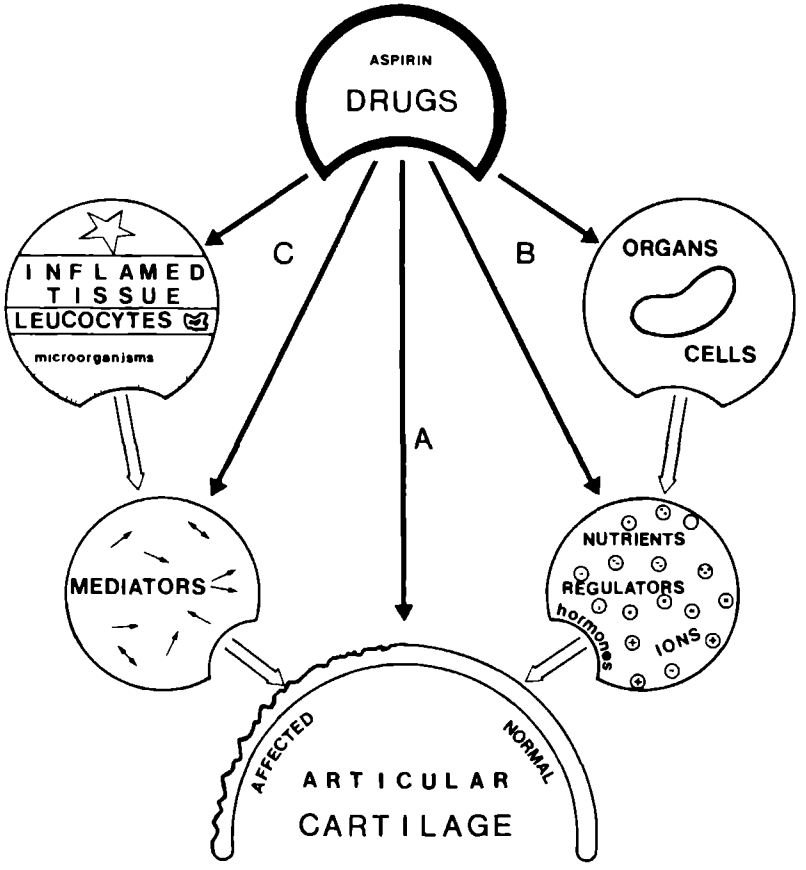
ASPIRIN
DRUGS
C
B
A
INFLAMED
TISSUE
LEUCOCYTES
microorganisms
ORGANS
CELLS
MEDIATORS
NUTRIENTS
REGULATORS
hormones
IONS
AFFECTED
NORMAL
ARTICULAR
CARTILAGE

Figure 4

C. An indirect effect by affecting production, release or activity of inflammatory mediators which influence chondrocytes or merely attack the extracellular matrix. Inflammatory mediators influencing cartilage metabolism are, for instance, arachidonic acid metabolites (prostaglandins, leukotriens) cytokines (interleukins, tumor necrosis factor, catabolin), oxygen metabolites and proteolytic and saccharolytic enzymes. Drug studies on this subject can be done in vitro (co-culture of cartilage with mediators, activated cells or inflamed tissue) as well as in vivo. In the latter case beneficial or deleterious effects of drugs cannot simply be ascribed to modulation of one mediator system. Of course in medical practice of patient treatment all 3 types of drug actions can play an important role. However, most experimental studies so far have mainly focused on actions A and C and surprisingly little attention has been payed to action B. Potential effects of drugs on nutrients and growth factors is a rather unexplored field in general and especially with respect to antiinflammatory drugs like the commonly used NSAIDs.

Screening of the various drug effects on cartilage, as mentioned above can be undertaken in vitro as well as in vivo, and a combination is obviously most informative. Studies on synthesis and breakdown of cartilage constituents are mainly focused on proteoglycans rather than collagen. When drugs are screened in in vitro systems, usually short-term experiments are performed and effects are quantitated using radiolabels like ^{35}S-sulfate or ^{3}H-glucosamine (see section 1.3.1 and Chapter 2). Such studies are aimed to unravel mechanistic actions of drugs. Long-term drug effects can be studied in vivo and radiolabel studies then have to be combined with histological and biochemical analysis (Chapter 3) of the overall composition and quality of the cartilage.

In vitro screening

Screening of direct drug effects on cartilage has been performed in a wide variety of culture systems and under variable conditions. Culture systems in use include: isolated chondrocytes (51,70,104) damaged car-

tilage explants like disks and slices (11,52,64,112) and anatomically intact cartilage specimens like limb buds (17), femoral heads (106), whole metatarsal bones (77), whole femoral condyles (78) and whole patellae (108,109, Chapter 2). Proper evaluation of effects obtained is furthermore hampered by variables like age of the cartilage (112,113), anatomical location the specimen is taken from (11,77,113), and intrinsic species differences. For instance, salicylate mediated suppression of GAG synthesis appeared to be markedly higher in rat compared to murine patellar cartilage (own unpubl obs). Moreover, salicylate caused a clear suppressive effect in habitually unloaded cartilage, whereas it did not affect loaded cartilage (11 and Chapter 9). By far most studies are done with normal cartilage, some data were reported on cartilage from experimental osteoarthrosis (11), whereas until recently (Chapter 7) no studies were done with arthritic cartilage.

The magnitude of a chondrocyte response upon direct action of antirheumatic drugs depends on the drug concentration ultimately reaching the cell as well as the chondrocyte susceptibility. Both variables are determined by environmental factors like extracellular matrix composition and culture conditions. Apart from already being affected in vivo by whatever cause (e.g. disease, trauma, degeneration, infection), cartilage, isolated from its natural environment, will ultimately possess chondrocytes with more or less deviations from their in vivo metabolic state due to:

1. withdrawal from systemic or local factors influencing chondrocyte metabolism directly or indirectly (pressure and joint motion).
2. disrupting or removing the cartilage matrix by making slices or isolating chondrocytes respectively.
3. keeping it alive in artificial culture conditions.

Drug concentration. In vivo more than 80% of most drugs are bound to plasma proteins; unfortunately this circumstance is almost never met in in vitro culture systems. Nothing is known about potentially different effects of protein-bound drug over free drug. Anyway the amount of free drug concentration in the culture medium is determined by the amount of drug-binding substances, whatever they are. De Vries et al (108, Chap-

ter 5) found a decrease in the degree of suppression of GAG synthesis in murine patellar cartilage if serum or albumin were put into the culture medium containing sodium salicylate, indicating that the free salicylate concentration reaching the chondrocytes is decreased owing to protein binding. In this respect it is important to note that the suppressive effect of therapeutic salicylate concentrations (10^{-3} - $2x10^{-3}$ M) was not obvious anymore in the presence of 25% serum, suggesting that salicylate will do no harm in vivo on healthy cartilage. Very low concentrations of salicylate and indomethacin are reported to even stimulate chondrocyte GAG synthesis (21,108). These observations suggest that the two drugs in vivo will exert merely a slight stimulating effect instead of the opined suppressive influence on articular GAG synthesis. Also the extracellular cartilage matrix on itself can influence the drug amount reaching the chondrocyte. Palmoski and Brandt (78) demonstrated that the uptake of salicylate and indomethacin was enhanced inside cartilage with decreased PG content. Consequently, isolated chondrocytes, devoid of any matrix, are exposed to much higher drug load than chondrocytes surrounded by a protective cartilage matrix.

Chondrocyte susceptibility: influence of the culture medium. An important factor in chondrocyte susceptibility is the composition of the culture medium. Besides the normal nutrients which can vary substantially in between commercially available media, extra supplements like vitamins, growth factors, antibiotics, serum and synovial fluid may affect chondrocyte function (Table 5). Moreover, additives like organic chemical solutes (e.g. ethanol, dmso, propylene glycol in which drugs sometimes are dissolved initially), supernatants from other cell, tissue or organ cultures or products liberated by these cultures, when introduced into the cartilage/chondrocyte culture system (co-culture), may lead to changes (reversible or irreversible) in chondrocyte metabolism. Chondrocytes might ultimately even dedifferentiate to other cell phenotypes (e.g. fibroblasts) attended with altered properties (8). Also factors like atmospheric composition (oxygen tension;15) mechanical and osmotic pressure schedules (44,52,93,97,104), temperature and incubation time (113) can contribute to these changes. Sometimes a check on synthesis of collagen type II (a characteristic marker for hyalin arti-

Table 5. Nutrients, factors and additives in culture medium, exerting a profound effect on chondrocyte GAG metabolism.

Medium component	References
Serum + growth factors	(3,80,83)
Conditioned medium factor (CM)	(98)
Oxygen tension	(15)
Nutrients:	
Glucose	(3,49,113,114)
D-glucosamine	(50,106)
L-glutamine	(3,113)
Serine	(3)
Anorganic ions:	
H_3O^+ (=pH)	(3,53,102)
Na^+, K^+, Mg^{2+}	(3)
Ca^{2+}	(73)
Mn^{2+}	(65,94)
$SO_4^=$	(53,93,108)
$PO_4^{\equiv}$	(53)
Vitamins:	
A	(68,81)
C	(53,83,95)
E	(95)
K	(54)
Antibiotic:	
Actinomycin D	(3)
Drug vehicles:	
Ethanol	(21)
Propylene glycol	(113)
Inflammatory mediators, like:	
Prostaglandins	(52,59)
Catabolin/Interleukin I	(85)
Tumor necrosis factor (TNF)	(88)
Other lymphokines	(39)
Proteases	(7)
Hydrogen peroxide	(91)

cular cartilage chondrocytes) is used to "identify" the cell phenotype (8), but notwithstanding the production of the right cartilage matrix components, variable responses towards drugs might be expected depending on which of the above mentioned conditions is met. Remarkably little investigations have been devoted to a possible relationship of chondrocyte metabolic condition and the magnitude of direct drug effects. Some data are available. For instance, we found substantial evidence that there seems to exist a threshold sulfate concentration above which, the sulfate incorporation rate into chondrocytic GAGs is maximal (107,108, Chapter 4 and 5). Below this threshold, the suppressive effect of sodium salicylate on murine articular GAG synthesis significantly increased as compared to the magnitude of suppression achieved at threshold sulfate concentrations. Ommission of sulfate from the medium, often done to obtain high radiosulfate incorporation values upon labeling with carrier-free $^{35}SO_4$, even more enhanced the suppressive effect, mediated by salicylate (108). The sulfate threshold varies for cartilage explants derived from different species and seems to correspond with their physiological sulfate concentrations in the circulation (69) i.e.: human, 0.3 mM (62,93), mice 1,0 mM (107), rat 0.8 mM (own obs), cow 1.2 mM (62). As far as we know our study is the only existing report, demonstrating a variation in chondrocyte susceptibility towards an antirheumatic drug, mediated by variation in the concentration of only one nutrient in the culture medium. It would be of interest to know if this phenomenon, described here for sulfate and the antirheumatic, salicylate, also exists for other nutrients and drugs. Another example of increased susceptibility is reported by Steinberg et al (99) who studied the effect of hydrocortisone on normal cartilage PG breakdown. They found that the greater the basal PG degradation rate, the more effectively it was inhibited by hydrocortisone, suggesting a higher susceptibility of the metabolically-changed chondrocytes to hydrocortisone.

The action of antirheumatic drugs can be more complex but will also be more adequate by introducing in the culture medium (direct or via co-culture) substances with high potency in modulating chondrocyte metabolic processes (e.g. hormones, cytokines, prostaglandins, vitamins; Table 5) or components possessing merely destructive properties, di-

rected in first instance to the cartilage extracellular matrix (e.g. enzymes, reactive oxygen metabolites; Table 5). The affected chondrocytes can be rendered more susceptible towards drugs, but also the action of these chondrocyte- and matrix affecting factors can be influenced by antirheumatic drugs by inhibiting or just enhancing production or activity of these factors. An example, concerned with affected chondrocytes and higher susceptibility is found in the literature (51). Articular chondrocytes, not sensitive to a relative low concentration of hydrocortisone (2.2×10^{-6} M), obtained a reduced GAG synthetic activity after exposure to conditioned-synovium-medium. The decreased GAG synthesis in these affected chondrocytes was suppressed still more if hydrocortisone was present in concentrations of $2.2x10^{-8}$-$2.2.x10^{-6}$ M.

Chondrocyte susceptibility: influence of cartilage composition and chondrocyte function. It is not precisely known if and to what extent the composition and condition of the extracellular matrix, besides providing variable access of drugs to the chondrocytes, influences the metabolic state of the chondrocytes and with it perhaps the degree of drug-susceptibility. In general a decrease in PG content will lead to an increased rate of PG synthesis (35). This situation was created in hyaluronidase treated canine cartilage slices (75); these slices demonstrated higher vulnerability to the action of salicylate (suppression of GAG synthesis) than normal cartilage slices did. Of interest is that, whereas these latter cartilage slices were affected to some extent by a therapeutic salicylate concentration (10^{-3} M), anatomically intact cartilage, from which the slices in question originated, did not respond to a similar salicylate exposure (11,78). Along this line an important observation was made with anatomically intact OA cartilage and atrophic cartilage, isolated from experimental dogs with unilateral affected joints (78). Both cartilage specimens had matrixes depleted of PG to a comparable extent (Table 6), but GAG synthetic rate in OA and atrophic cartilage was significantly increased and decreased, respectively, as compared to control values (contralateral joint). Assuming an almost similar drug uptake by these cartilages, the only relevant aspect is a difference in chondrocyte metabolic state. Upon exposure of

Table 6. Effect of sodium salicylate (10^{-3} M) on articular cartilage in the presence of 10% serum.*

Articular cartilage	% PG content	% GAG synthesis		Ref
		control	salicylate	
1. Anatomically intact, normal:	100	100	100	(78)
2. Anatomically intact, OA:	50	150	15	(78)
3. Anatomically intact, atrophic:	65	50	40	(78)
4. Slices of 1:	100	100	79	(75)
5. Slices of 1, treated with hyaluronidase:	60	132	64	(75)

*Taken from Palmoski and Brandt.

these cartilages to therapeutic concentrations of salicylate (10^{-3} M) or indomethacin (4×10^{-6} M), GAG synthesis was suppressed profoundly in OA cartilage while only moderately in atrophic cartilage, indicating that OA chondrocytes were more susceptible to these NSAIDs than "atrophic" chondrocytes. Recent data, however, suggest that this phenomenon is only apparent when the chondrocytes are embedded in their natural surrounding (affected) cartilage matrix, since differences in drug-susceptibility disappeared when isolated chondrocytes were examined (12). So it might be that the cartilage matrix, in whatever condition, is a potential factor in determining the susceptibility of chondrocytes towards antirheumatics (Table 6). This knowledge is important for in vitro studies and necessitates future research on this issue.

The discussion above leaves us with a dilemma with respect to a proper choice of a relevant in vitro system for drug screening. On the one hand it seems reasonable to perform short-term in vitro studies with anatomically intact cartilage. On the other hand long-term drug exposure is more like the in vivo situation, but bears the substantial risk of culture induced changes of cartilage integrity and potential changes in chondrocyte susceptibility.

In vivo screening

No doubt, in vivo drug studies are of utmost relevance to test an eventual harmful or beneficial effect on articular cartilage. In vitro methods can impossibly cover all in vivo facets influencing and regulating chondrocyte metabolism, nor the pathophysiological processes present in arthropathies. A number of comments made in the previous section about chondrocyte susceptibility in vitro also apply to the in vivo situation. Apart from direct effects on cartilage, drugs may exert indirect effects in vivo, by interfering with endogenous regulating factors and by suppressing the inflammatory process (see Figure 4). The further discussion will be mainly restricted to the last two items.

Since experimental in vivo studies cannot be performed on healthy subjects or patients, at the least because of the lack of adequate controls, experimental animals are needed. To avoid interindividual differences in outcomes, experiments are often performed with inbred strains. In this respect only small animals, like mice, rats, guinea pigs (and rabbits) are considered; sometimes dogs are used because their physiology is more comparable to humans. In addition, many experimental models exist of several kinds of arthropathies, resembling pathological features found in rheumatoid patients. Frequently, these joint disorders can be induced in a reproducible way with predictive magnitudes of severity and duration, allowing reliable drug studies in which articular cartilage is the main topic. The most convenient way to obtain information about in vivo drug effects on normal and pathological cartilage is to work with experimental models with unilaterally induced knee joint disorders, for example the antigen induced arthritis model (40,55) and the various experimental arthrosis models (19,23,61, 77,96). The potential dual action of drugs in the antigen induced arthritis model is depicted in Figure 5. As a consequence of joint inflammation, synthetic function of the chondrocyte is already suppressed (9) and relief of joint inflammation will indirectly have a beneficial effect on chondrocyte function. On the other hand, drugs may exert a direct harmful effect on the chondrocyte and this effect may potentially be enhanced in already damaged cartilage (see in vitro screening section). The usefullness of a drug in the treatment of arthritis de-

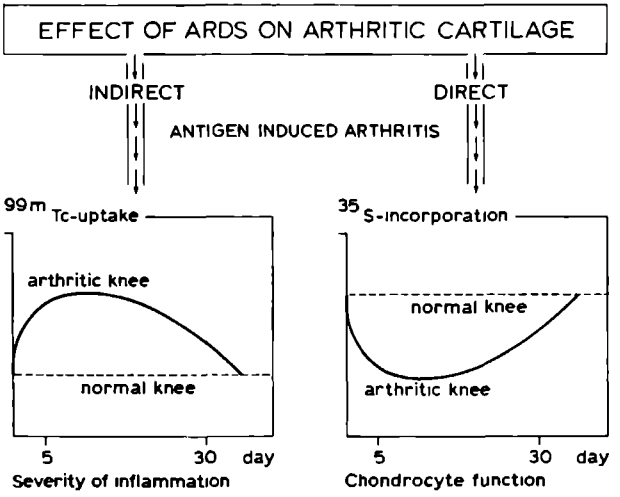

Figure 5. The potential dual action of antirheumatic drugs.

pends on its overall action, on both inflammation and cartilage. Upon drug treatment its effect can be screened by measuring the severity of the inflammation (^{99m}Tc-uptake, 55) and the chondrocyte synthetic activity (radiolabel incorporation, section 1.3.1) either in vivo or ex vivo. The latter method is less expensive in terms of radiolabel use, but may yield different results (see forecoming section about systemic drug effects and pitfalls). Upon long-term drug treatrent radiolabel studies have to be combined with biochemical analysis of the cartilage. When drug effects are not that impressive, electron microscopy has proven to be a valuable tool to detect alterations in chondrocyte function (4).

Effect on endogenous regulating factors. Aside from effects on cartilage or the inflammatory process, drugs may directly influence the supply and turnover of endogenous factors like nutrients and hormones (Table 7A). Also along this line indirect effects have to be considered since inflammation may damage endogenous mediators (e.g. by oxygen radical attack) and antiinflammatory drugs may suppress this event. Our

Table 7A. Drug mediated disturbance in the supply or turnover of nutrients and hormones, affecting chondrocyte function in vivo.

Drug	Humoral factor	References
Glucocorticoids	glucose, insulin, K^+	(26,79)
Sodium salicylate*	SO_4	(92,107)
Salicylamide	$SO_4^=$	(31)
Paracetamol	SO_4	(69)
Calcitonin*	$SO_4^=$,$PO_4^{\equiv}$,Ca^{2+}	(63,89)
Arteparon	hydrocortisone	(13,32)
Vitamin A	$SO_4^=$	(92)
Tolbutamide	SO_4	(92)
Triiodothyronine	$SO_4^=$	(92)
Rape-seed oil#	$SO_4^=$	(92)

*Sulfaturic effect. #Used as a drug vehicle.

Table 7B. Some drugs, serving as substrate for sulfate conjugation.

Drug	References
Salicylamide	(29,69)
Flufenamic acid	(10)
Paracetamol	(69)
Phenylbutazone	(10,29)
Oxyphenbutazone	(10)
Prednisolone	(29)
Cortisone	(29)
Hydrocortisone	(10)
Most corticosteroids	(69)
Chloroquine diphosphate	(10,29,30)
Hydroxychloroquine	(29)
Most phenols	(69)
Vitamin C, D	(69)

interest was especially drawn to effects of drugs on endogenous sulfate levels (Chapter 4 and 5). Chondrocyte synthesis of sulfated GAGs is highly dependent on the sulfate level (107) and our in vivo observations with salicylate point to interference at this level. Shortly after salicylate administration to mice a deep fall in endogenous sulfate was seen and this was probably responsible for the observed suppression of chondrocyte sulfated GAG synthesis (107,108). Some drugs may influence endogenous sulfate by a diuretic effect (Table 7A) or by serving as a substrate for sulfate conjugation (Table 7B).

Farmacokinetic of drugs. Drug concentration, exposure time, chondrocyte susceptibility and reversibility of drug effects all determine the magnitude of a potential side effect of drugs on articular cartilage in vivo. The drug concentration reaching the chondrocyte is determined not only by the amount of drug binding substances in the circulation and composition of the cartilage matrix, but also by absorption and elimination kinetics. Consequently chondrocytes are exposed to a gradient of drug concentrations. Since some drugs may exert different effects at various concentrations this aspect must be born in mind with in vivo (radiolabel) studies.

Pitfalls in experimental in vivo drug studies. Drug administration: To achieve the same therapeutic "window" of drug concentrations in plasma of man, corresponding with antiinflammatory properties, most animal species need different doses of a drug (mg/kg) compared to man, for their pharmacokinetics with respect to that drug differ (Table 8). For instance, in mice 200 mg sodium salicylate per kg body weight was needed to achieve temporary a plasma concentration of 1-2 x 10^{-3} M salicylate (107), a therapeutic window for humans (57). In man this window is settled after intake of only 50 mg sodium salicylate/kg (31). This means that in mice some organs, dependent on the route of administration and the absorption- and elimination kinetics, are overloaded with this drug (e.g. stomach, liver, kidneys). Consequently, different from the human situation, a potential drug effect on articular cartilage in animals might be, more or less, attributable to indirect effects, exerted by the drug affected organs which, for example, disturb the

Table 8. Doses of antirheumatic drugs in humans compared to those in mice.*

Antirheumatic drug	Oral dose (mg/kg)		
	man	mouse	mouse/man
Sodium salicylate	50	200	4
Ibuprofen	6-9	50-100	8-11
Flurbiprofen	0.7-1.5	1-9	1.5-6
Indomethacin	0.4-1.4	1-3	2-2.5
Phenylbutazone	1.4	15-50	10-35
Prednisolone	0.1-0.6	1-10	10-17

*Summarized from refs: 31,41,87,107,116.

normal nutrient supply towards cartilage.

Drug metabolites: Biotransformation of (pro)drugs (conjugation, hydrolysis) may be different in various species, and metabolites of drugs may be more or less potent than the original substance.

Radiolabel studies: Optimally, one should like to be informed about the actual chondrocyte metabolic activity, at the moment of maximal drug effect. Short-term label studies (15 - 30 minutes) are most appropriate for this goal, but such experiments are very expensive and ungracious to the milieu (high amounts of radiolabel needed). Moreover, the time span of maximal effect after a single drug dose does not necessarily have to synchronize with that of maximal drug concentration. Usually, a 2 - 24 h labeling period is chosen, starting at the same time or shortly after drug administration (10,16,107). When double-label studies are performed, e.g. to obtain information about effects on synthesis of GAG backbone (^{14}C- or ^{3}H-glucosamine) and GAG sulfation (^{35}S-sulfate), another problem is met. The incorporation kinetics of the two radiolabels are rather different, resulting in discrepancies in labeling intensity of the two labels in just synthesized sulfated GAGs (50,109). This holds, of course, also for in vitro studies.

Salicylate causes a drastic fall in endogenous sulfate shortly after

administration to mice and, dependent on the moment of ^{35}S-sulfate injection, this may result in changes in the specific activity ($^{35}SO_4$/SO_4) in drug treated versus control animals. As a consequence ^{35}S incorporation values must be corrected for these changes in specific activity (36,107). It seems that numerous investigators (e.g. 16,85 and refs cited in 36) did not pay attention to this aspect in their in vivo ^{35}S-sulfate studies. These problems, encountered with salicylate are of utmost relevance and should always be kept in mind when screening a new drug.

Upon long-term drug treatment the proper choice of the time period to screen chondrocyte metabolic function is a matter of considerable debat. In our studies with mice (Chapter 8), the animals were treated with drugs one or two times a day (9 h a.m. and 6 h p.m.). At the end of a 4-8 weeks drug treatment one should like to be informed about the overall status of the chondrocyte and not about the effect related to the last drug dose. On the other hand, when the period between the last drug injection and administration of radiolabel is too long, relief of potential suppression may occur and the chondrocyte will try to restore the balance, resulting in an overshoot phenomenon. These latter issues are also involved in ex vivo studies, after drug treatment in vivo, (and might also occur in mere in vitro drug studies).

Stress: Another practical problem encountered with (chronic) drug dosing to experimental animals is the potential stress caused by e.g. oral gavage. Recently, experiments were started to compare arthritis in "stressed" and resting animals but conclusive data are not yet available (Chapter 8). If stress leads to changes in steroid levels (6), the arthritis may already be suppressed to some extent thereby preventing potential beneficial effects of administered antiphlogistic drugs.

Evaluation of the presented information leads to the conclusion that joint cartilage, and no other connective tissue, should be used to screen potential drug effects. Moreover it is necessary to examine also cartilage resembling arthrotic or arthritic cartilage, since during drug therapy we have to face pathological joint cartilage. Studies focused on chondrocyte metabolism should include anabolic as well as catabolic processes in addition to quality control of matrix constituents.

Most reliable information ought to be revealed by **in vivo** studies, not the least because of drug-induced indirect effects on joint cartilage. Since one has the opportunity to make use of several relevant animal OA and RA models, antirheumatic drugs should be tested herein. Some investigators like Colombo et al (19), Maier and Wilhelmi (61), Hunneyball et al (40-42), and Palmoski and Brandt (77) already performed more or less long-term drug exposure studies in these kind of animal models. The advantage of short-term in vivo drug experiments is that they can give rapid information about drug modulation of cartilage PG metabolism and properties. It should be a matter of future research to perfect these short-term studies, by preventing pitfalls like those mentioned above, to obtain an in vivo model with real predictive value concerning ultimate cartilage damage. Actually, results obtained with drugs in inbred strains should be reinvestigated in animals with more human-like physiology and (cartilage) properties (e.g. dogs), before any reliable conclusion can be made in respect to drug action towards human cartilage during medication.

In vitro studies have the advantage of being relatively cheap, and may serve to elucidate a potential mechanism of drug action. With regard to the many variables influencing chondrocyte metabolism (section 1.3.2) and (perhaps) drug susceptibility, results should be interpreted with caution. Culture conditions resembling the physiological environment of joint cartilage of the animal strain in question are difficult to realize, but should be approached as much as possible. This holds also for co-cultures with other tissues. Culture medium with the same composition as the synovial fluid would already be a good alternative

for an in vivo condition. Furthermore, it is important to use drug concentrations in the range of therapeutic plasma values to avoid toxic effects. Chondrocytes should not be isolated because their "natural" drug susceptibility is probably changed compared to chondrocytes embedded in the (pathological) cartilage matrix. This on its turn should preferably not be extra damaged by preparing slices or disks. Since the in vivo condition never can be met in vitro (e.g. absence of the right pressure rhythm, humoral regulating factors), articular chondrocytes might change their behaviour, and (perhaps) drug susceptibility, with increasing incubation time. Thus, short-term studies (hrs), immediately performed after isolation of fresh explants of anatomically intact joint cartilage, are most informative. If under these conditions a real effect, harmless or harmful, of drug on animal cartilage is evident it should at least be reexamined, in vitro, on human (pathological) cartilage.

1.5 AIM OF THE PRESENT INVESTIGATION

The main purpose of our study has been to elucidate the potential side effects of NSAIDs on articular cartilage. The first Chapters (2,3) deal with optimizing a screening system for effects of drugs on articular cartilage GAG metabolism. In Chapter 2 the suitability of various radiolabels is shown for the measurement of GAG synthesis and its sulfation in murine patellar cartilage. In Chapter 3 a sensitive method is described to enable sulfate determinations in small serum and cartilage samples.

Acetylsalicylic acid (aspirin) is in many contries the NSAID of choice in the management of rheumatoid arthritis. In these contries the use of newer NSAIDs is only justified if they are demonstrably more effective, less toxic or better tolerated than aspirin. This drug is considered as the prototype of NSAIDs and used in many experimental drug studies as a reference drug. Our studies of drug effects on cartilage were also started with salicylate, both in vivo (Chapter 4) and in vitro (Chapter 5). A comparative study with tiaprofenic acid is described in Chapter 6. Since drugs are normally given to OA or RA patients who have already damaged cartilage, the following Chapter (7) was aimed to elucidate differences in drug-susceptibility of normal, artificially damaged, and arthritic cartilage. This was performed both in vitro and in vivo using short-term drug exposure. Finally, long-term drug effects were determined in an animal model of rheumatoid arthritis, namely antigen-induced arthritis (Chapter 8). Since this study has not been completed yet, tentative results are only briefly discussed.

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CHAPTER 2

QUANTITATION OF GLYCOSAMINOGLYCAN METABOLISM IN ANATOMICALLY INTACT ARTICULAR CARTILAGE OF THE MOUSE PATELLA: IN VITRO AND IN VIVO STUDIES WITH ^{35}S-SULFATE, ^{3}H-GLUCOSAMINE, AND ^{3}H-ACETATE

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Quantitation of glycosaminoglycan metabolism in anatomically intact articular cartilage of the mouse patella: in vitro and in vivo studies with ^{35}S-sulfate, ^{3}H-glucosamine, and ^{3}H-acetate

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Summary. We investigated the usefulness of the whole mouse patella to quantitate the synthesis of the glycosaminoglycan (GAG) backbone and its sulfation by intact murine articular cartilage both in vitro and in vivo Using ^{35}S-sulfate ^{3}H-glucosamine or ^{3}H-acetate as precursors of GAG synthesis it was found that more than 90% of the incorporated radioactivity was confined to the patellar cartilage layer compared to the whole patella Overnight papain digestion was enough to liberate more than 95% of the incorporated radiolabels except for ^{3}H-acetate for which 15–25% was not digestable Comparison of radio activity in the patella and that in quantitatively isolated GAGs revealed that for ^{35}S sulfate incorporation studies the whole patella can be used as a reliable measure for sulfated GAG synthesis The situation was different for the GAG backbone precursors ^{3}H-glucosamine and ^{3}H acetate more than 50% of the ^{3}H labels were incorporated into compounds other than GAGs or non covalently associated with matrix components Hence in studying GAG-backbone metabolism polysaccharides must be isolated quantitatively from cartilage In vivo studies made it clear that both ^{35}S-sulfate and ^{3}H-glucosamine are incorporated into patellar GAGs in amounts high enough to enable proper quantitation and that the route of administration (intraperitoneally or intravenously) is of minor importance Due to its low specificity for cartilage GAGs, ^{3}H-acetate is not suitable for such studies

Key words Articular cartilage – Glycosaminoglycans ^{35}S-sulfate ^{3}H-glucosamine – ^{3}H-acetate

Introduction

The metabolism of articular cartilage glycosamino glycans (GAGs) can be studied using various radiolabels ^{35}S-sulfate is the one most frequently used as precursor of GAGs [1–4] Strictly speaking this radiolabel only gives information about the sulfate metabolism of sulfated GAGs This seems of importance since it has previously been shown that the synthesis of the GAG polysaccharide chain and its sulfation are uncoupled phenomena [5] Un coupling can be demonstrated using low sulfate concentrations [6 7] Recent observations from our laboratory suggest that the same phenomena may occur during drug treatment of mice Salicylate was shown to induce a significant depletion of endogenous sulfate resulting in a low serum sulfate concentration and this was held respon sible for the observed decrease in synthesis of sulfated GAGs [8 9]

Another important point which deserves attention is the intactness of the articular cartilage matrix Many in vitro studies are performed with cartilage slices i e damaged cartilage to investigate the effects of inflammatory mediators or drugs Upon cultivating these cartilages tend to loose proteoglycans (PGs) mainly at the sliced sides [10 11] Since it has been shown that drugs exert a greater effect on chondrocytes in a PG depleted matrix [12] it is necessary to investigate undamaged cartilage in these kinds of studies Recently an easy and fast cartilage assay was developed in our laboratory [13 15] using the patella of the mouse as a source of anatomically intact articular cartilage The usefulness of this assay has been demonstrated for ^{35}S-sulfate and it has been used in several mediator studies [8 16 18] In the present investigation we extend the suitability of this assay by showing that both GAG backbone synthesis and its sulfation can reliably be quantitated in vitro as well as in vivo

Materials and methods

Animals Male 6–8 week old C57Bl10 mice in good health and ranging in weight from 20 25 g were used The animals were maintained on a commercial pellet diet tap water was provided ad libitum

In vitro studies Mice were sacrificed using ether anesthesia followed by cervical dislocation Whole patellae with a standard amount of surrounding tissue were dissected from the knee joints and assayed as described by van den Berg et al [13] In brief they were placed in RPMI culture medium (200 µl/patella) containing – in addition to the usual ingredients (Flow laboratories Irvine Scotland) – gentamycin (50 µg/ml) and L glutamine (2 mM) Hepes as well as bicarbonate (ratio 1 1) served as buffer in

maintaining the pH at 7 4 After a preincubation period of 2 h at 37 °C under 5% CO_2 atmosphere the patella specimens were transferred to fresh medium with two radioactive GAG precursors (each 20 µCi/ml) and incubation was continued for an additional 2 h Double label experiments were carried out with $Na_2{}^{35}SO_4$ (1 Ci/mmol) and D 6 ^{3}H glucosamine hydrochloride (40 Ci/mmol) or the sodium salt of ^{3}H acetate (300 mCi/mmol) (all purchased from Radiochemical Centre Amersham UK)

Effects of cycloheximide salicylate and oxygen metabolites Cycloheximide and sodium salicylate known inhibitors of protein and GAG synthesis respectively [8 19] were used for in vitro studies These drugs were present during the whole incubation period (i e 2 h preincubation followed by 2 h label incorporation)

Oxygen derived metabolites (superoxide anion and hydrogen peroxide) were generated by a xanthine xanthine oxidase (XaXaOx) system and patella specimens were exposed to XaXaOx for 45 min as recently described [17] Subsequently these patella specimens were washed and radiolabeled as described above

In vivo studies Groups of mice were injected intravenously or intraperitoneally with ^{35}S and ^{3}H labels simultaneously $Na_2{}^{35}SO_4$ (50 mCi/mmol 2 µCi/g) and ^{3}H glucosamine (8 µCi/g) or ^{3}H acetate (8 µCi/g) ^{3}H specific activity as stated above Radioactivity was taken up in 0 15 M NaCl and administered at 10 µl/g body weight to the animals At various time intervals after injection of radiolabel mice were sacrificed and whole patellae with surrounding tissue were removed and analyzed Blood samples (10 µl) obtained from the tail vein were taken at different times after radiolabel administration and lysed in 0 5 ml of water

Autoradiography Radiolabeled patella specimens fixed in 10% formalin and decalcified in 5% formic acid were embedded in paraffin Seven µm sections were prepared and mounted on gelatin coated slides These were dipped in K5 emulsion (Ilford Basildon Essex UK) and exposed for 3 weeks Slides were developed and stained with hematoxylin and eosin (HE)

Isolation of whole patellae In vitro or in vivo radiolabeled patella specimens were washed in 0 15 M NaCl (twice 10 min) fixed for 1 h in 10% formalin (pH 7 0) supplemented with 0 2% cetyl pyridinium chloride and decalcified overnight in fixative saturated with Na_2 EDTA Some patella specimens then were treated with acetone for 1 h in order to remove fatty substances Finally all tissues were transferred to formalin again In every step 3 ml liquid was used per patella This procedure removed all unbound radiolabel and in addition enabled the whole anatomically intact patella to be punched out easily from the surrounding tissue Both the punched out patellae and the tissues left behind were analyzed for radioactivity content (see below)

Isolation of patellar cartilage To remove the cartilage layer quantitatively from the underlying bone patella specimens were fixed in ethanol (96%) instead of formalin for at least 1 hour followed by decalcification in 5% formic acid overnight The patellae were punched out from the adherent tissue and placed under a stereo microscope Using tweezers the whole cartilage layer could be stripped off quantitatively from the subchondral bone as was confirmed by histology

Isolation of glycosaminoglycans A papain mixture (pH 6 0) a modification of that described by Adams and Muir [20] consisting of 1 mg/ml papain (Merck Darmstadt FRG) 0 2 M NaCl 0 1 M Na acetate 10 mM L cysteine hydrochloride 50 mM Na_2 EDTA and 50 µg GAG carrier/ml (chondroitin 6 sulfate chondroitin 4 sulfate hyaluronic acid = 3 3 1) was used to digest formalin fixed whole patellae (200 µl/patella) at 60 °C overnight Undigested patellar remnants were spun down To 150 µl supernatant of papain digest was added 150 µl 0 2% cetylpyridinium chloride (CPC) After 2 h at 37 °C the precipitate (CPC GAG complexes) was centrifuged at 10 000 *g* for 30 min at room temperature The supernatant was discarded and the pellet was treated once with 300 µl 0 05% CPC to remove small amounts of adherent radioactivity

Measurement of radioactivity Whole patellae extra patellar tissues cartilage layers as well as CPC GAG complexes were solubilized overnight in 500 µl Solulyte (J T Baker Chemicals Deventer The Netherlands) at 60 °C and subsequently taken up in 10 ml Lipofluor scintillation fluid (J T Baker Chemicals) The water lysed radioactive blood samples were mixed with 10 ml Aqualuma plus (Lumac/3M Schaesberg The Netherlands)

Tritium and ^{35}S radioactivity were counted in a liquid scintillation spectrometer (Philips PW 4700) (^{3}H window 0 4 8 keV ^{35}S window 15 160 keV) The channel overlap from the ^{35}S to the ^{3}H channel was about 15% and reversed it was about 1% Appropriate corrections were made for these overlaps There was no need for quench correction since tissues and CPC GAG complexes had the same quenching as measured by automatic external standardization Blood samples on the other hand had a constant shift owing to quenching as compared with the other samples Radioactive contents were expressed as counts per minute (cpm)

Results

Measurement of GAG synthesis in patellar cartilage Whole patellae with surrounding tissue were incubated with ^{35}S sulfate ^{3}H glucosamine and ^{3}H acetate as precursors of GAGs The incorporation patterns in the various structures of the patella specimen were determined with autoradiography Compared with the non labeled patella in Fig 1 clear black granules above the patellar cartilage were seen with all three radiolabels (Figs 2 4) Hardly any radioactivity was found in the underlying bone and bone marrow cells Note the absence of epiphyseal cartilage in the patella Radiolabel was also found in the patellar plate and at the insertion sites of ligamentous structures attached to the patellar bone Significant amounts of ^{3}H glucosamine and ^{3}H acetate were also found in the surrounding connective tissue and the latter in particular in fat tissue Quantitative data on the partition of the various labels over the patella and the surrounding tissue are shown in Table 1

In addition we separated the cartilage layer from the underlying bone of the patella For all three radiolabels more than 90% of the radioactivity found in the whole patella appeared to be confined to this cartilage layer (Table 1) This indicates that the radioactive content of the patella can be used as a reliable measure for the amount of radioactivity in the cartilage at least for the labels in question

To determine the amount of radiolabel incorporated into GAGs isolated patellae were first digested with papain followed by precipitation of the radiolabel with cetylpyridinium chloride (CPC) Overnight papain digestion appeared to be sufficient to release more than 95% of ^{35}S and ^{3}H glucosamine but 15–25% ^{3}H acetate remained associated with undigested patellar remnants Nearly all the incorporated ^{35}S-sulfate (94%) could be precipitated

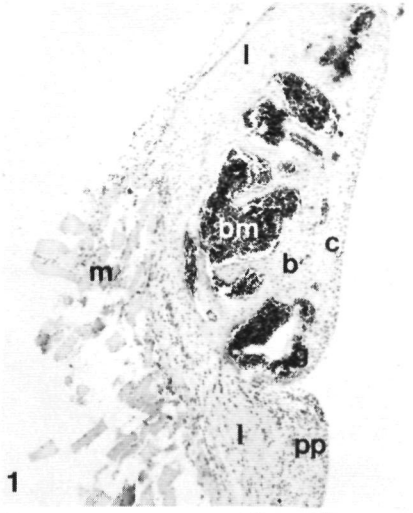
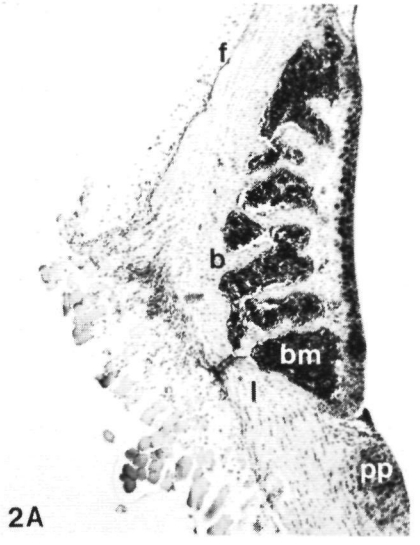


Fig. 1. Section of a mouse patella surrounded by connective tissue. Notice the absence of epiphyseal cartilage. × 40. HE staining. c = cartilage; b = bone; bm = bone marrow; pp = patellar plate; l = ligament; f = fat tissue; m = muscle

Fig. 2 A, B. Autoradiograph of a section of a patella specimen labeled with ^{35}S-sulfate in vitro. HE staining. Abbreviations as in Fig. 1
A Cartilage and the patellar plate (cartilaginous, extra-patellar tissue) contain incorporated radioactivity. Hardly any incorporation is detectable in other tissue structures. × 40.
B Detail of ^{35}S-labeled patella; × 400. Notice the heavily labeled chondrocytes in the cartilage and the absence of label in subchondral bone (s) and bone marrow



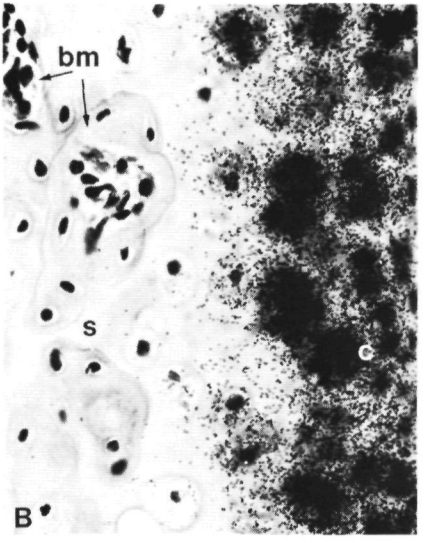


Table 1. Incorporation of radiolabel in patella specimens

Radiolabel	Patella (cpm)	Tissue (cpm)	Patella/tissue (%)	Cartilage/patella[a] (%)
^{35}S-sulfate	1 659 ± 274[b]	2 252 ± 552	42	99
^{3}H-glucosamine	906 ± 92	6 875 ± 1 818	12	94
^{3}H-acetate	1 618 ± 205	60 151 ± 19 109	3	92

[a] Patella specimens, from a separate experiment, were fixed in ethanol and decalcified in 5% formic acid. Cartilage was removed completely from the subchondral bone and radio-content was compared with that of similarly treated, whole patellae
[b] Values represent the mean ± SD for six mice

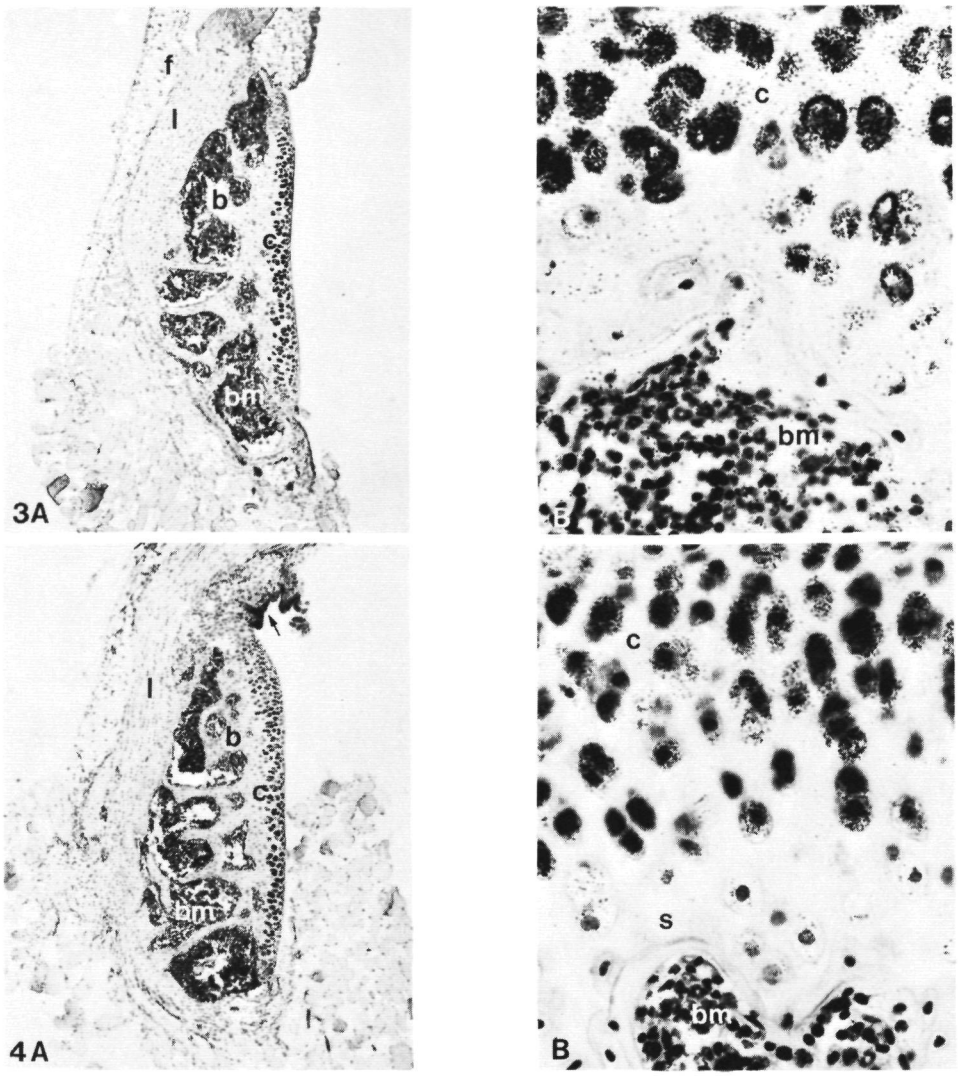


Fig. 3 A, B. Autoradiograph of a section of a patella specimen labeled with ^{3}H-glucosamine in vitro. HE staining. Abbreviations as in Fig. 1. **A** Label is almost exclusively present in cartilage. ×40. **B** Detail of ^{3}H-glucosamine-labeled patella; ×400. Besides labeling in the chondrocytes some radioactivity is incorporated in bone marrow cells

Fig. 4 A, B. Autoradiograph of a section of a patella specimen labeled with ^{3}H-acetate in vitro. HE staining. Abbreviations as in Fig. 1. **A** A substantial amount is incorporated in the chondrocytes, but label is also found in fat tissue attached to the ligament and in the synovial lining (arrow). ×40. **B** Detail of a ^{3}H-acetate labeled patella; ×400. The cartilage chondrocytes are clearly labeled and almost no radioactivity is present in the subchondral bone (s) and bone marrow

Table 2. Incorporation of radiolabel in whole patellae and isolated patellar GAGs. Effect of cycloheximide[a]

Radiolabel	Cycloheximide (0.1 mM)	Patella (cpm)	GAGs (cpm)	GAGs/patella (%)
^{35}S-sulfate	–	1 595 ± 344	1 504 ± 286	94
	+	101 ± 9 (6%)[b]	91 ± 11 (6%)	
^{3}H-glucosamine	–	953 ± 198	457 ± 61	48
	+	472 ± 89 (50%)	51 ± 10 (11%)	
^{3}H-acetate	–	1 753 ± 344	301 ± 53	17
	+	1 565 ± 391 (84%)	22 ± 9 (7%)	
^{3}H-acetate (degreased)	–	1 305 ± 324	323 ± 75	25
	+	907 ± 118 (74%)	24 ± 5 (7%)	

[a] Data represent the mean ± SD of patellae obtained from five mice
[b] Percentage of cpm incorporated in cycloheximide-treated patellae compared with controls

with CPC, whereas for ^{3}H-glucosamine and ^{3}H-acetate the precipitable radioactivity appeared to be much lower (Table 2).

Since autoradiography revealed that large amounts of ^{3}H-acetate were incorporated in fat tissue (Fig. 5) and the cartilage matrix and chondrocytes also contain fatty substances, we performed an additional experiment using acetone pretreatment. Double-labeling with ^{35}S-sulfate demonstrated that this procedure had no effect on the digestion efficiency and precipitation of GAGs. Degreasing with acetone had only a small effect on the amount of ^{3}H-acetate retained in whole patellae (Table 2); the amount of precipitable label (GAGs) remained unchanged.

Table 2 further shows the effect of cycloheximide on label incorporation. Initiation of proteoglycan-GAG synthesis has been described as protein-core dependent [21] and almost complete inhibition by cycloheximide (0.1 mM) was found for the CPC-precipitable material, in contrast to minor effects on the bulk of ^{3}H-label incorporated in the patellar cartilage. In addition, CPC precipitations were performed under various ionic strengths. Compared to the amount of radioactivity precipitated at 0.15 M NaCl, the standard salt concentration used throughout this study, about 95% of ^{3}H-labeled material was still precipitable between 0.2 M and 0.8 M NaCl. Significantly less precipitation (less than 50%) of both ^{3}H- and ^{35}S-labeled material occurred at 0.95 M NaCl. This pattern of ionic strength dependence indicates that ^{35}S-sulfate and the majority of the ^{3}H-label are incorporated in sulfated GAGs and that, probably because of a low metabolic turnover rate, hyaluronic acid does not play an important role in these label studies.

Effect of oxygen metabolites and salicylate. Patellae were exposed to a xanthine-xanthine oxidase (XaXaOx) system or sodium salicylate followed by incubation with ^{35}S-sulfate and ^{3}H-glucosamine (double-label). The incorporation of ^{3}H-glucosamine into the whole patella or isolated GAGs was inhibited to the same extent by XaXaOx treatment (62% and 59% respectively; Table 3) indicating that oxygen metabolites impaired the overall synthetic activity of the patellar chondrocytes. Salicylate, however, appeared to selectively suppress the chondrocyte GAG synthesis. The incorporation of ^{3}H-glucosamine in the whole patella was hardly affected, in contrast to the clear inhibition found with isolated GAGs (Table 3). No differences were observed between the decreased amounts of ^{35}S incorporated in the whole patella or isolated GAGs.

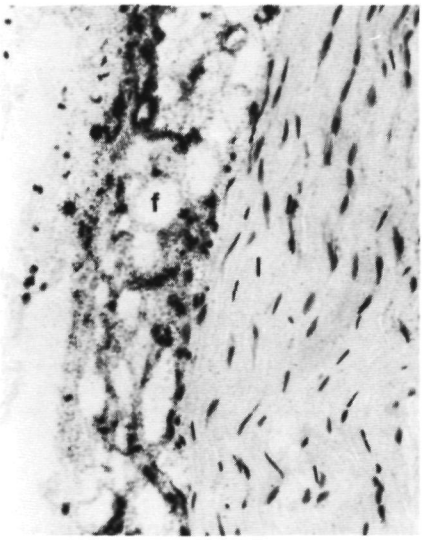


Fig. 5. Autoradiograph of a section of extra-patellar fat tissue labeled with ^{3}H-acetate; ×250. Fat tissue (f) attached to the ligamentum patellae (1) contains ^{3}H-activity, whereas label is almost absent in the ligament

Glycosaminoglycan synthesis in vivo. To investigate the usefulness of the three radiolabels for in vivo incorporation

Table 3 Effects of oxygen metabolites and salicylate on the incorporation rate of radiolabel in whole patellae and in isolated GAGs[a]

Radiolabel	Analyzed	Control (cpm)	XaXaOx[b] (cpm)	SodSal (cpm)
^{35}S sulfate	Patellae	1 529 ± 234	785 ± 198 (51%)[c]	534 + 102 (35%)
	GAGs	1 435 + 318	696 ± 216 (49%)	551 ± 126 (38%)
^{3}H glucosamine	Patellae	1 025 + 180	633 ± 131 (62%)	840 ± 134 (82%)
	GAGs	467 ± 88	276 ± 66 (59%)	274 + 58 (59%)

[a] Values represent the mean ± SD of patellae obtained from six mice
[b] XaXaOx = xanthine xanthine oxidase oxygen metabolites generating system SodSal = sodium salicylate See Materials and methods for details
[c] Percentage of control

Table 4 Incorporated radiolabel 2 h after intravenous administration of ^{35}S sulfate ^{3}H glucosamine and ^{3}H acetate[a]

Radiolabel	Dose (μCi/g)	Patella (cpm)	GAGs (cpm)	GAGs/patella (%)
^{35}S sulfate	2	388 ± 31	369 ± 57	95
^{3}H glucosamine	8	1 233 ± 82	321 ± 64	26
^{3}H acetate	8	267 + 59	33 ± 9	12

[a] Cpm values represent the mean ± SD for five mice

studies mice were injected with high doses of the various labels either intraperitoneally (i p) or intravenously (i v) Table 4 shows the incorporation values in patellae at 2 h after i v administration

Both ^{35}S sulfate and ^{3}H glucosamine seem suitable for studying sulfated GAG and GAG backbone synthesis The absolute amount of ^{3}H acetate recovered in isolated patellar GAGs is however too low The ratio between the incorporated amount of ^{3}H label in the isolated GAGs and in the whole patella as observed in vitro (Table 2) was reduced in vivo (Table 4) The incorporation kinetics of ^{35}S sulfate and ^{3}H-glucosamine are shown in Fig 6 for i p administration of the labels Maximal incorporation was already achieved at 2 h for ^{3}H glucosamine whereas for ^{35}S-sulfate a significant increase was still observable between 2 and 4 h The blood kinetics are shown in Fig 7 The ^{35}S blood values steadily decreased after 15 30 min while the ^{3}H values clearly increased after 30 min These phenomena were seen both after i p (Fig 7A) and i v (Fig 7B) administration Although small differences exist in the blood kinetics of the labels after i p or i v administration in particular in the first 15 min no large differences were found in the amounts of radiolabel incorporated in patellar GAGs

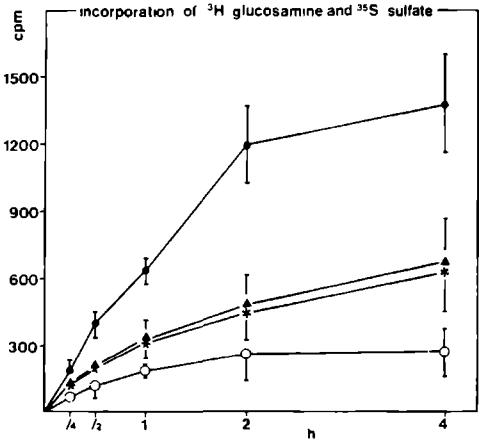


Fig 6 Sulfur 35 sulfate and ^{3}H glucosamine contents of whole patellae and of isolated patellar GAGs at different points of time after intraperitoneal administration of 2 μCi ^{35}S sulfate and 8 μCi ^{3}H glucosamine per gram body weight Data represent the mean + SD from groups of five mice ● = Tritium content in whole patellae = ^{3}H content in GAGs isolated from contralateral patellae ▲ = ^{35}S content in whole patellae * = ^{35}S content in GAGs isolated from contralateral patellae

Discussion

Cartilage GAGs except hyaluronic acid are the polysaccharide chains of proteoglycans immense molecules which together with collagen and water constitute the cartilage ground substance GAGs are also present though to a lesser extent in bone and bone marrow structures also present in the patella The average metabolic turnover in these structures is however much lower compared to cartilage as the results of this study clearly show that after exposure of patellae to ^{35}S-sulfate ^{3}H glucosamine or ^{3}H acetate these radiolabels almost exclusively incorporate into the articular cartilage layer of the patella (Figs 2–4 Table 2)

Isolation of GAGs by means of CPC precipitation of papain digested cartilage is a well known standard method [22] The almost complete recovery of ^{35}S label in CPC-precipitates derived from papain digests of ^{35}S labeled patellae indicates that patellar GAGs can be isolated quantitatively in this way

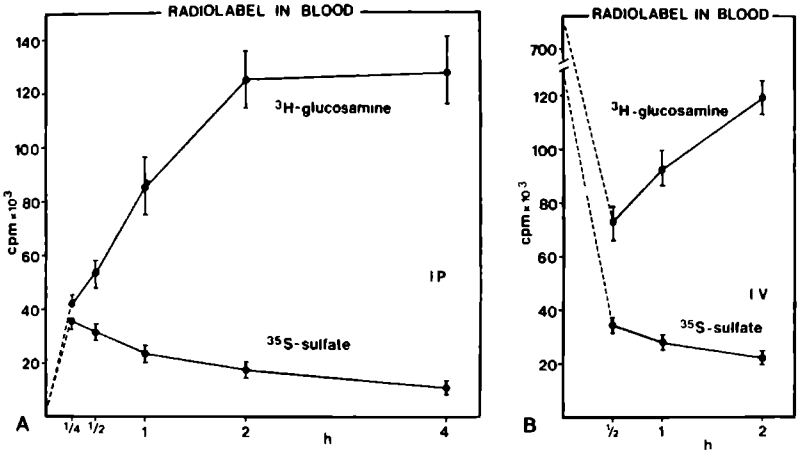


Fig. 7 A, B. Sulfur-35-sulfate and ^{3}H-glucosamine contents in 10 µl blood samples obtained at various times after administration of 2 µCi ^{35}S-sulfate and 8 µCi ^{3}H-glucosamine per gram body weight. Data represent the mean ± SD of blood samples obtained from five mice. **A** Blood kinetics after intraperitoneal injection. **B** After intravenous injection.

Degreasing cartilage prior to protease digestion is thought to be better for GAG release from matrix proteoglycans [23]. Except that the wash out of ^{3}H-acetate from the patella specimens was much faster with acetone as degreasing agent than with aqueous solutions, we found no difference in the ultimate ^{3}H-GAG yield. Neither the wash out of ^{3}H-glucosamine or ^{35}S-sulfate nor the yield of GAGs marked with these radiolabels, was affected by acetone treatment.

We have no information about the overall GAG composition in murine patellar cartilage. According to the definitions of Scott [22] about effects of ionic strengths, nearly all the radiolabel in CPC-complexes of GAGs isolated from the patella represented sulfated glycosaminoglycans, whereas the contribution of ^{3}H-labeled hyaluronic acid (HA) was less than 5%. If cycloheximide is added to the patella culture system the incorporated radioactivity in CPC-complexes is much decreased (above 90%). This indicates that GAGs were precipitated since synthesis of GAGs, except HA, was shown to be protein synthesis-dependent [21, 24]. Moreover, because the turnover rate of HA is not influenced by cycloheximide [25] its formation can be neglected as judged by the low ^{3}H-incorporation in the presence of this drug. Above all, these observations demonstrate that simple diffusion of label becoming attached to precipitable GAGs can be ruled out.

Sulfur 35-sulfate appeared to be highly specific for sulfated GAGs, since other cartilage constituents – not precipitable with CPC after papain digestion – were not significantly labeled with ^{35}S-sulfate, as can be concluded from the data in Table 2. In contrast, ^{3}H-glucosamine a precursor for glucosamine as well as galactosamine containing GAGs [26, 27], and ^{3}H-acetate are less specific in that they also incorporate in biomolecules other than articular GAGs (i.e. CPC-precipitable material) or are bound non-enzymatically to matrix components. Less than 50% of the radiocontent of the whole, punched-out patella was associated with GAGs (Table 2). The lower specificity for GAGs is also illustrated by the relatively high uptake of the latter two radiolabels in non-cartilaginous extra-patellar tissue (Table 1 and Figs 3, 4). Since glucosamine is known to be a characteristic constituent of glycoproteins [28] and acetate is the starting material for the synthesis of many biomolecules the above observations are not unexpected.

Presumably a substantial fraction of the amount of ^{3}H-glucosamine and ^{3}H-acetate present in the cartilage of a radiolabeled patella, is incorporated in the large UDP-N-acetylhexosamine pool of the chondrocytes, a pool with a slow turnover rate through which the radiotracers have to pass before they can enter the GAG backbone chain [29]. Apart from the incorporation of the ^{3}H labels in non-GAG compounds, the presence of such a pool, which is probably difficult to wash out [6] also stresses the need for papain digestion followed by GAG precipitation. On the other hand, ^{35}S-sulfate is very rapidly conjugated to the GAGs of proteoglycans via the intracellular 3'-phosphoadenosine-5'-phosphosulfate (PAPS) pool, which has a high turnover rate and, moreover, was found to be present in very small amounts [29]. From this point of view there is no need to isolate sulfated GAGs. Almost all the ^{35}S label in cartilage is theoretically expected to be incorporated into GAGs, as in fact is found with our patella assay.

These considerations further demonstrate that if radiolabeling is performed with a double label (^{35}S and ^{3}H) it is important to make allowance for the differences in

incorporation kinetics when studying proteoglycan metabolism As outlined ^{35}S-sulfate is incorporated almost immediately in sulfated GAGs whereas ^{3}H-glucosamine incorporation proceeds with a time lag [29 30] Therefore uncertainty exists whether incorporation of ^{3}H and ^{35}S takes place in the same GAG molecules and moreover information is lacking about the specific activities of ^{3}H GAGs [26] This knowledge would be of value in studies of undersulfation of GAGs (see below)

Examining the inhibitory effects of SodSal on in vitro GAG synthesis revealed that ^{35}S incorporation in isolated GAGs as well as in the whole patella obviously is more suppressed than the incorporation of ^{3}H-glucosamine into patellar GAGs (Table 3) This phenomenon has been observed by us in previous studies [31] and is also reported to occur in other connective tissues [32 33] We feel that detailed analyses of the GAGs in question should be performed before conclusions can be drawn about undersulfation It is furthermore shown by our experiments that short-term exposure to oxygen metabolites impairs the overall patellar chondrocyte synthetic activity to a significant degree Not only is GAG synthesis affected but also the synthesis of UDP-N-acetylhexosamines seemed impaired since ^{3}H glucosamine uptake in the whole patella was equally suppressed as the ^{3}H incorporation into isolated GAGs (Table 3)

In vivo GAG synthesis was investigated by measuring the amount of incorporated radiolabel injected i v or i p Comparing the data in Table 4 with those of Fig 6 demonstrates that the amount of incorporated radioactivity in patellae is hardly influenced by the route of label administration Tritiated-acetate appeared to be unsuitable for in vivo studies since only a very small fraction (33 cpm) of the high dose given (8 μC/g) was incorporated in patellar GAGs We do not know whether this low uptake is due to a relatively low specific activity of ^{3}H acetate ultimately reaching the articular chondrocytes Maximum labeling was already found 30 min after i v injection (data not shown)

The kinetics of incorporation of i p administrated ^{35}S sulfate and ^{3}H-glucosamine in whole patellae and in quantitatively isolated patellar GAGs were examined over a period of 4 h After 2 h maximum incorporation of ^{3}H-glucosamine into patellar GAGs was achieved whereas ^{35}S-sulfate incorporation still continued to increase As shown in vitro, ^{35}S-sulfate incorporation in the whole patella behaves in a similar way to the incorporation in patellar GAGs indicating that on both occasions ^{35}S is associated with the same molecular entities Of note the ratio of ^{3}H labeled GAGs over ^{3}H-labeled patellae appeared to decrease with time We have no explanation for this phenomenon The ^{3}H blood content increased during the exposure time of mice to this label administrated i p or i v (Fig 7A B) Since maximum incorporation of i p ^{3}H-glucosamine was found at 2 h the high amounts of ^{3}H label found in the blood between 2 and 4 h cannot represent ^{3}H-glucosamine These observations are consistent with the findings of Shetlar et al [34] who studied the fate of i p administered ^{14}C glucosamine in the rat Maximum ^{14}C-content in the serum appeared 3 h after injection and represented 24% of the total injected dose However already within 2 h as little as 1% of the original injected dose represented still free ^{14}C-glucosamine The increasing ^{14}C-content was attributable to labeled serum glycoproteins produced by the liver Apparently a similar situation exists in the mouse As determined in recent studies [8] ^{35}S sulfate in serum represented more than 95% inorganic sulfate over the whole period studied

Finally we also did some preliminary experiments with ^{3}H-glucose another cheap and frequently used GAG-precursor which not only incorporates into hexosamines but also into the uronic acid moieties of GAGs In vitro incorporation characteristics much resembled those of ^{3}H-acetate (data not shown) Since the incorporation kinetics of glucose into GAGs is still more complicated as is reported for glucosamine [26] we did not make further use of this label

In summary ^{35}S-sulfate is a good precursor for quantitative studies on the sulfation of sulfated GAGs Isolation of ^{35}S-GAGs is not necessary since the radiosulfate content of the anatomically intact patella appeared to be exclusively associated with sulfated GAGs in the cartilage On the other hand ^{3}H glucosamine proved to be a suitable label for GAG backbone metabolism both in vitro and in vivo Unfortunately this precursor is not specifically incorporated into GAGs and hence quantitative isolation of GAGs is required Because there is no epiphyseal cartilage present in the patella this assay is particularly convenient for metabolic studies on articular and moreover anatomically intact cartilage There is no need to quantitate the content of cartilage constituents or determine tissue weights as routinely done in most assays Whether or not GAGs are isolated results can simply be expressed as radioactivity content per whole patella

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CHAPTER 3

DETERMINATION OF SMALL QUANTITIES OF SULFATE (0-12 nmol) IN SERUM, URINE AND CARTILAGE OF THE MOUSE

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ABSTRACT

Using disposable 96 well-microplates instead of individual cuvettes the colorimetric benzidine method of Dodgson and Spencer (1953) for the measurement of inorganic sulfate, could be scaled down about 100 times. Ten µl samples of serum and urine, derived from mice, could be analysed in a simple, rapid and reliable way without the need to sacrifice the animals. Without prior isolation of sulfated glycosaminoglycans, ester sulfate in mouse patellar cartilage was liberated quantitatively as inorganic sulfate upon acid hydrolysis in 3 M HCl for 16 h at 80 °C. To this end the articular cartilage layer of the patella had to be separated in toto from the underlying bone. Subsequent hydrolysis in polypropylene tubes gave accurate results. In contrast hydrolysis in borosilicate glass vials turned out to be useless, since nanomoles sulfate added could not be recovered adequately. The thin patellar cartilage layer obtained from male mice, 10 weeks of age, contains about 5 nmol sulfate, an amount easily measured with the developed microplate benzidine method.

INTRODUCTION

Certain drugs are known to have an effect on the sulfate contents of mouse serum, urine and cartilage. Recently we obtained evidence that sodium salicylate, administered to mice, decreased inorganic serum sulfate levels to such an extent that the concurrently observed inhibition of sulfated glycosaminoglycan synthesis in patellar cartilage could be related to this(1). In this particular study a turbidimetric method was used (2) for the determination of inorganic sulfate in serum and urine. Although sulfate measurements were satisfactory, mice had to be killed in order to obtain enough serum (150-500 µl) for analysis. To be able to study serum sulfate kinetics and ester sulfate contents in cartilage of animals as small as mice, there was a need for a quick and easy sulfate assay with a low detection limit. We did not succeed to scale down the turbidimetric method, originally described by Berglund and Sörbo (3) and thereafter modified by others (2,4). A number of other, highly sensitive methods are reported (detection limits<20 nmol SO_4), based on different techniques (5-13). However, none of them appeared useful to us since they either are too laborious, gave no reproducible results or make use of expensive analytical instruments not present at our laboratory. We herein describe the application of a scaled down version of the spectrophotometric benzidine method as reported by Dodgson and Spencer (14). Using disposable ELISA microplates instead of cuvettes large numbers of samples containing small amounts of sulfate can readily be measured. Special attention is paid to the acid hydrolysis of cartilage, necessary to measure the ester sulfate content. The modified assay appeared simple, rapid, reliable and practical for our research purposes, (e.g. only 10 µl serum is needed for sulfate analysis).

MATERIALS AND METHODS

Samples. Serum and urine were obtained from C57Bl10 male mice, about 10 weeks of age. Prior to analysis urine was always diluted 50 times with deionized and distilled water. Articular cartilage was obtained from mouse patellae. To be able to remove the cartilage layer quantitatively from the underlying bone, ethanol (96%) fixed (≥ 1 hr) patellae, with still adjacent surrounding tissue, were decalcified in 5% formic acid

overnight. After punching the whole patellae out of the adherent tissue, the wet, intact cartilage layer could be stripped of from the subchondral bone using a stereomicroscope and forceps (see Figure 5 and 6).

Sulfate determination method. All solutions were handled and made in plastic (usually polypropylene or polystyrene), disposable labware; deionized and distilled water was used throughout the analytical procedure. Procedure: 10 or 15 µl aliquots of serum or diluted urine are placed in Eppendorf microtubes (0.5 ml) with or without 5 µl deionized water respectively, and 60 µl 6.25 % trichloroacetic acid (TCA; Merck, Darmstadt, FRG), a protein precipitating agent not affecting inorganic sulfate (15), is added (final concentration 5 %). The tube contents are centrifugated for 15 minutes at 10,000 x g. Supernatants are subsequently analysed. Fifty µl aliquots of standard solutions (0-0.24 mM Na_2SO_4 in 5 % TCA), supernatants or 5 % TCA solutions of dried cartilage hydrolysates (see below) are placed in Eppendorf microtubes (1.5 ml). Hundred and twenty-five µl of benzidine reagent (0.5 % benzidine in 96 % ethanol; Sigma, St Louis, USA) is added and the mixture is allowed to stand 1 hour at -20 °C followed by centrifugation at 4 °C for 30 minutes at 10,000 x g. The supernatant is carefully decanted and discarded; 450 µl aceton-ethanol (1:1) is added followed by recentrifugation. After again careful decantation (draining by inverting tubes) mouths of the mirotubes are wiped with tissue paper to remove last traces of aceton-ethanol (all free benzidine must be removed!). The precipitate (benzidine-sulfate) is dissolved in 75 µl 1 M HCl and allowed to stand 30 minutes at room temperature. Benzidine is diazotized by the addition of 75 µl 0.033 M $NaNO_2$. After 5 minutes, 125 µl thymol reagent (0.5 % thymol in 2 M NaOH; Merck, Darmstadt, FRG) is added and a red coloured product, stable for at least 4 hour, will appear. Extinction of 100 µl volumes is read at 492 nm in polystyrene ELISA microtiter plates, consisting of 96 U-shaped wells (Greiner, Nurtingen, FRG), with a Titertek Multiscan Spectrophotometer (Flow laboratories, Irvine, Scotland). Sulfate concentrations are calculated from a standard curve.

Hydrolysis of cartilage. Patellar cartilage layers were hydrolysed in 3 M HCl (100 µl/cartilage layer) in closed polypropylene microtubes (1.5 ml) for 4-72 hour at 80 °C. Hydrolysates were dried under vacuum and

taken up in 60 µl 5 % TCA. Fifty µl aliquots were analysed for sulfate content as described above.

Recovery experiments. Serum and urine, were depleted from SO_4 and subsequently reconstituted with Na_2SO_4 to test recovery. Upon exact determination of the inorganic sulfate concentration in pools of serum and diluted urine, with an established turbidimetric method (2), stoichiometric amounts of $BaCl_2$ were added. $BaSO_4$ precipitate was allowed to form at 4 °C overnight and subsequently removed by centrifugation at 10,000 x g for 30 minutes at 4 °C. With this procedure the sulfate concentration decreased to below 0.1 mM, but was still detectable with our modified benzidine method indicating that no free Ba^{++} was present. Preceeding to the sulfate recovery experiments known amounts of Na_2SO_4 were added to the $BaCl_2$-treated fluids; in the case of cartilage, Na_2SO_4 was added to 3 M HCl prior to hydrolysis. For ^{35}S recovery studies, patellae were labeled in vitro with $^{35}SO_4$ conform a described method (16).

Gag depleted cartilage. To obtain cartilage with decreased GAG contents some patellae, still embedded in surrounding tissue, were treated with trypsin (1 mg/ml RPMI 1640 medium) for two hours, or patellae were derived from zymosan induced arthritic mice (17). GAG contents were estimated with a dimethylmethylene blue dye-binding assay as described by Farndale et al. (18).

Statistical analysis. Correlation curves were fit by the least-squares method, and, where appropriate, mean data were compared by a two-tailed Student's t-test.

RESULTS

Analysis of standard sulfate solutions. Since there always was some difference (<5%) in optical density between the individual (empty) wells of a microplate, the absorbance of the red coloured thymol conjugate was taken as the mean of readings in two wells. The whole microplate (96 wells with 100 µl each) could be measured within one minute by a multiscan spectrophotometer. A typical standard curve for different amounts of inorganic sulfate, as measured with the scaled down

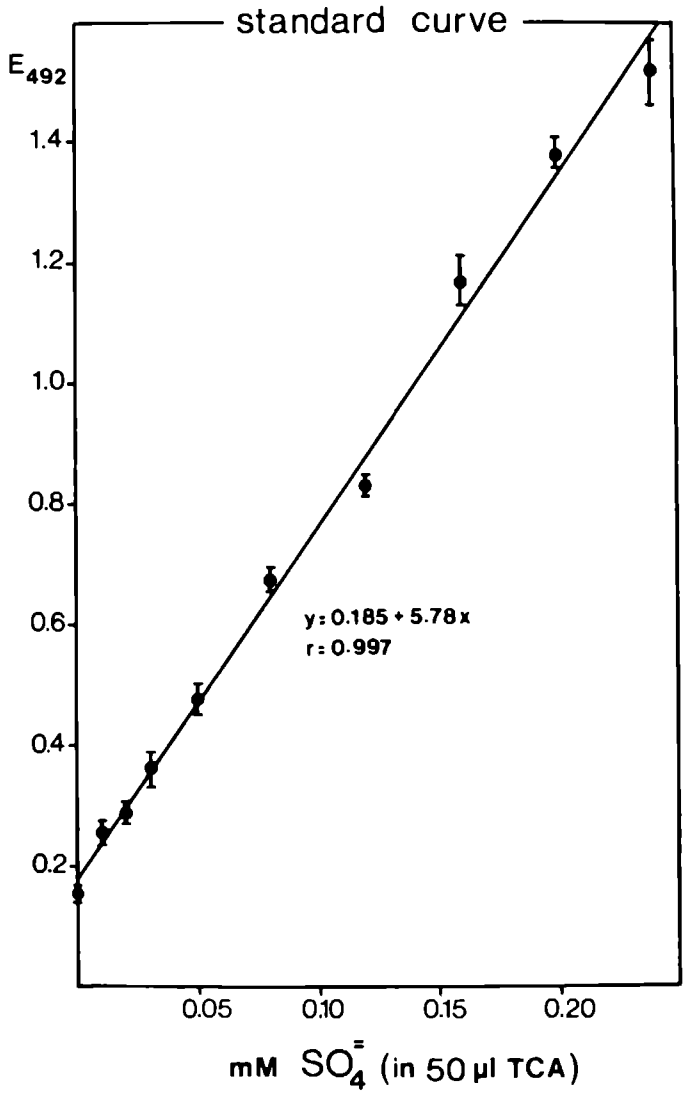

Figure 1. Relationship between the absorbance (492 nm) of the thymol-conjugate product, measured in indidual wells of a 96-well microplate, and the sulfate concentration in 50 µl samples containing 5% TCA. Line of best fit is shown, calculated from 10 sulfate determinations. Correlation is significant. Each value represents the mean ± SD of three measurements.

benzidine method in microplates, is shown in Figure 1. Lineair correlation of concentration (0-0.24 mM) and absorbance (492 nm) was obtained with standard samples of 50 µl in which TCA was present in a final concentration of 5 % (w/v); the absolute amount of measured sulfate ranged from 0-12 nmol. Since, as a contaminant, a small amount of sulfate is present in the TCA, we currently use, the regression line in Figure 1 does not pass through the origin. Measurement of sulfate in distilled water containing various amounts of Na_2SO_4 by the benzidine method correlated well with the results found by the established turbidimetric assay, in which Ba^{++} ions are used as sulfate precipitating agents (Figure 2). About 3% of the original sample volume (500 µl), needed for the turbidimetric method (2), suffices the benzidine method. The first method could not be scaled-down by using microplates, since the turbidity could not be measured adequately in the micro wells.

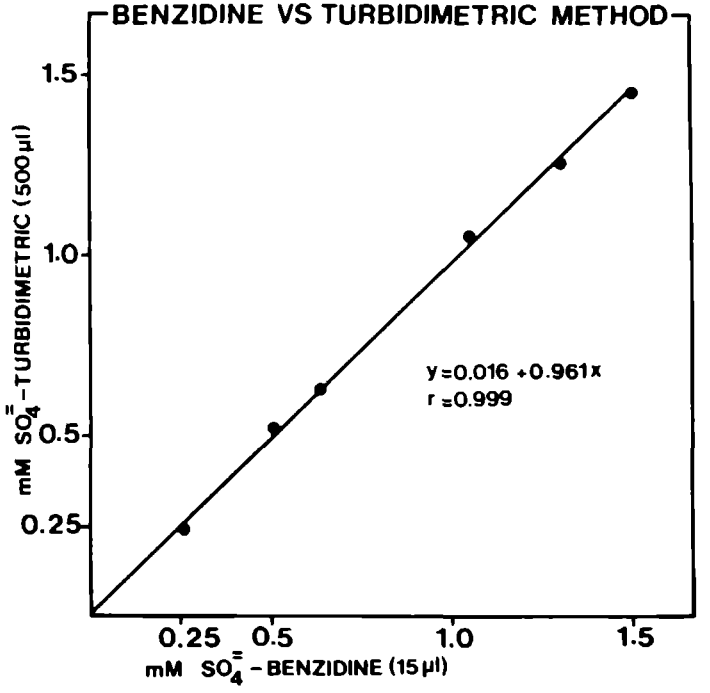

Figure 2. Correlation between results of sulfate determinations in 6 water samples by the microplate benzidine method and the established turbidimetric method. The regression line depicted is not significantly different from the line of identity.

Recovery of added sulfate from serum and urine. Preceeding to analysis of endogenous sulfate in normal serum and urine we investigated the recovery of added sulfate to test the reliability of the benzidine method for such body fluid samples. To this end mouse serum and urine were treated with $BaCl_2$ to remove specifically inorganic sulfate (see the Materials & Methods section). In this way, except for sulfate, the composition of these biological fluids remains almost unaffected. Since it was already known that mouse serum and urine contained approximately 1 mM and 50 mM SO_4 respectively, serum and urine samples (the latter always diluted 50 times) were reconstituted with Na_2SO_4 to obtain sulfate concentrations in the range of 0 - 1.2 mM. As can be seen in Figure 3 for serum as well as in Figure 4 for diluted urine, recovery of added inorganic sulfate from 10 µl samples was complete. These results indicate that constituents, normally present in serum and urine, do not disturb the measurement of sulfate by our benzidine method. To illustrate applications of this analytical method, small amounts (10 µl) of serum and diluted urine, obtained from mice, were analysed for inorganic sulfate content (Table 1). Also the effects of sodium salicylate and paracetamol on the serum sulfate concentration in mice were tested. In addition, serum and saliva, obtained from healthy humans, and serum and synovial fluid from rheumatoid arthritis patients were analyzed (Table 1). The measured sulfate concentration in normal mouse (1.04 mM) and human serum (0.33 mM) are identical with formerly reported values. Saliva, apparently, has the same sulfate concentration as is found in serum. In rheumatoid arthritis patients the serum and synovial fluid sulfate concentrations were essentially identical and did not differ from serum values obtained from healthy subjects. The antirheumatic drugs these patients took, at different times before sample collection, apparently had no effect on the sulfate level. Sodium salicylate as well as paracetamol significantly decreased the sulfate concentration in mice, a phenomenon also described by others using different sulfate detection methods. The very high sulfate concentration in urine was already corroborated by analysis with the turbidimetric method we formerly used.

Ester sulfate content in patellar cartilage. The relative huge amount of sulfate in cartilage tissue is covalently bound as ester sulfate to glycosaminoglycans and to a negligible extent to other matrix molecu-

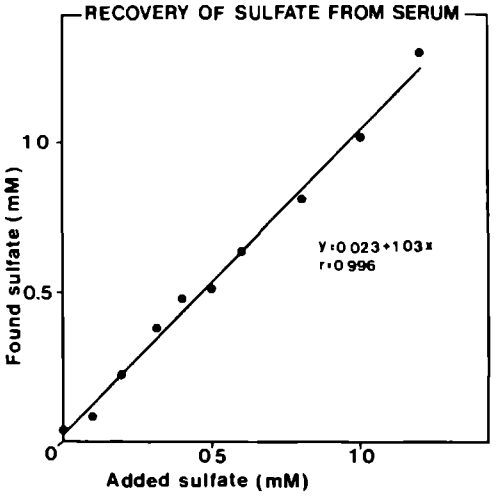

Figure 3. Recovery of inorganic sulfate added to sulfate depleted mouse serum. The regression line, calculated from 10 sulfate determinations, has a slope not significantly different from unity.

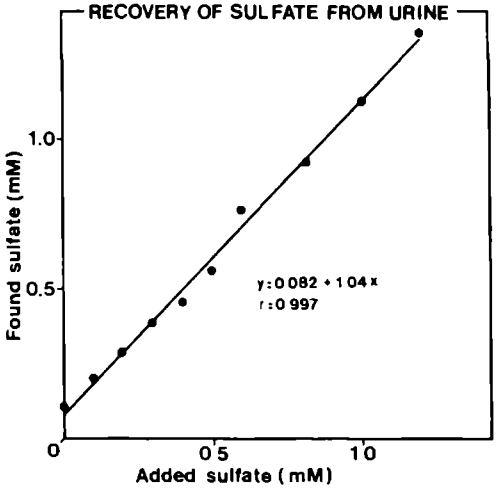

Figure 4. Recovery of SO_4 added to sulfate depleted and diluted (50 times) mouse urine. The regression line, calculated from 10 sulfate determinations, has a slope not significantly different from unity.

Table 1. Inorganic sulfate in serum, synovial fluid, saliva and urine§.

Species	Sample	Treatment	mM SO_4 (mean±SD)	(n)°
Mice	Serum	-	1.04 ± 0.05	(6)
	Serum	sodium salicylate	0.62 ± 0.08#	(6)
	Serum	paracetamol	0.65 ± 0.13#	(6)
	Urine	-	44.1 ± 6.0	(6)
Humans				
Healthy*:	Serum	-	0.33 ± 0.05	(20)
	Saliva	-	0.31 ± 0.10	(20)
RAΔ:	Serum	antirheumatics	0.303 ± 0.030	(5)
	Synovial fluid		0.295 ± 0.008	(5)

§ Sulfate assay applied on 10 µl samples; prior to analysis urine was diluted 50 times with deionized and distilled water.
° n = number of samples obtained from n mice or humans.
Samples were taken 4 hours after oral administration of 200 mg of each drug per kg body weight. $p<0.001$ as compared to serum values of untreated mice.
* Samples obtained from human volunteers, 20-35 years of age and of either sex. Saliva was collected 15 minutes after subjects had thoroughly rinsed their mouths with tap water.
Δ Samples obtained from 5 patients suffering from definite rheumatoid arthritis (RA), 42-61 years of age and of either sex. These patients were regularly taking a number of concurrent medications, but none of these were drugs known to be disturbing sulfate levels.

les. Mostly acid hydrolysis is performed upon which the liberated inorganic sulfate can be quantitated. We applied this procedure to articular cartilage and bone derived from the mouse patella. Ethanol as fixative and 5% formic acid as decalcifier enabled excellent separation of first the whole patella from the natural surrounding tissue (Figure 5) and second the quantitative separation of the cartilage layer from the underlying bone (Figure 6). Acid hydrolysis of these patella specimens, performed in disposable borosilicate glass vials (2 ml) with teflon-lined (srew-) caps, gave some problems. Vials purchased from several suppliers (e.g. Wheaton, Chrompack, Pierce) leaked variable amounts of absorbing material (2-5 nmol sulfate equivalents) whereas those from another supplier (ATS) just diminished the amount of (added) inorganic

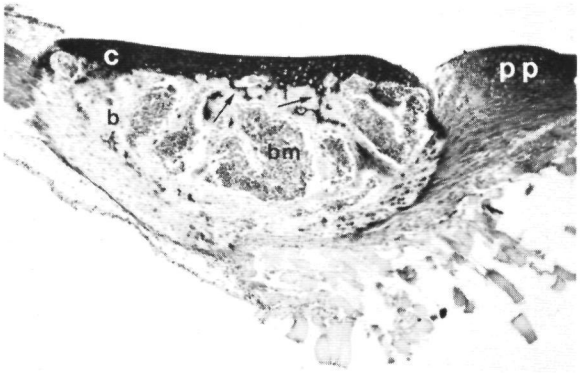

Figure 5. Safranin O staining of a section of a mouse patella embedded in natural surrounding tissue. Only the cartilage layer, covering the patellar articular side, with some processes penetrating the underlying subchondral bone (arrows) is stained, indicating that no other cartilaginous tissue is present in the patella. Original magnification 40 x. c = cartilage; b = bone; bm = bone marrow; pp = patellar plate (cartilaginous, extra-patellar tissue).

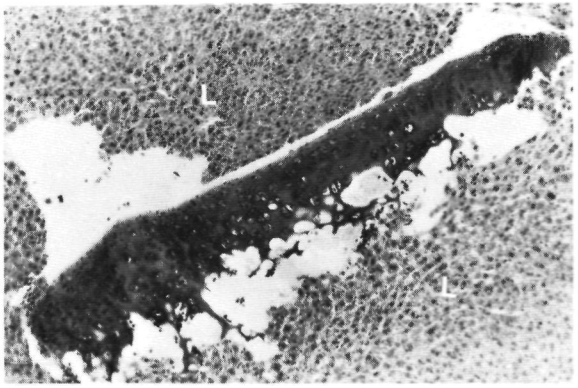

Figure 6. Safranin O staining of a section of a quantitatively removed cartilage layer from a mouse patella (see Figure 5), embedded in liver tissue (L). For male mice, 10 weeks of age, the estimated, average cartilage thickness is 0.1 mm and its (wet) weight about 80 µg; inorganic sulfate release, after acid hydrolysis, is circa 5 nmol (Table 4). Originial magnification 100 x.

sulfate (nmol range) as ultimately measured by 492 nm scanning of microplates (Table 2). These phenomena were seen with acids (3 M HCl and

Table 2. Recovery of inorganic sulfate added to 3 M HCl after standing overnight at 110 °C in borosilicate glass vials from several suppliers.

Sample (100 µl)	Added SO_4 (nmol)	Found SO_4 -equivalents (nmol)*		
		Wheaton	Chrompack	ATS
3 M HCl	0	2.7 ± 3.9	4.5 ± 1.9	0
	2	nd	7.8 ± 1.2	nd
	4	7.5 ± 3.5	9.5 ± 2.5	1.3 ± 0.6
	6	9.8 ± 3.0	11.1 ± 1.8	4.1 ± 1.6
Distilled, deionized water		nd	2.1 ± 0.9	nd

* Results represent the mean ± SD of six to ten sulfate analysis; nd = not determined.

5% TCA) as well as with water and both at elevated (110 °C) and moderate temperatures (about 60 °C). Consequently, after hydrolysis (>60° C) in borosilicate glass vials, measurement of small quantities of ester sulfate such as present in the cartilage of one mouse patella (≈5 nmol; see below) cannot be performed adequately. Polypropylene Eppendorf microtubes (1.5 ml) can be heated overnight at 80 °C without significant evaporation of 3 M HCl. We tried to use these tubes for acid hydrolysis experiments. Recovery of added sulfate was complete and sufficiently accurate (coefficient of variance <15%) from 3 M HCl with and without patellar cartilage layers (Figure 7).

Examination of the time of hydrolysis needed to liberate maximal amounts of inorganic sulfate revealed that 16 hours seems enough for the cartilage layer and patellar bone (Table 3). Since the sulfate content in bone accounted for about 20% of that of the whole patella, cartilage should be separated from bone prior to analysis.

To be sure that the whole content of glycosaminoglycan (GAG) sulfate in the patellar cartilage could actually be liberated as inorganic sulfate by acid hydrolysis, additional experiments were performed. Normal and GAG-depleted patellae were analysed for GAG contents, estimated as described (18) and expressed in chondroitin 4-sulfate units, and ester-sulfate contents, measured with our version of the benzidine method. Trypsin treatment resulted in a similar loss (41 %) of both sulfate and GAGs (Table 4). Also patellae derived from mice with 3 day-lasting zy-

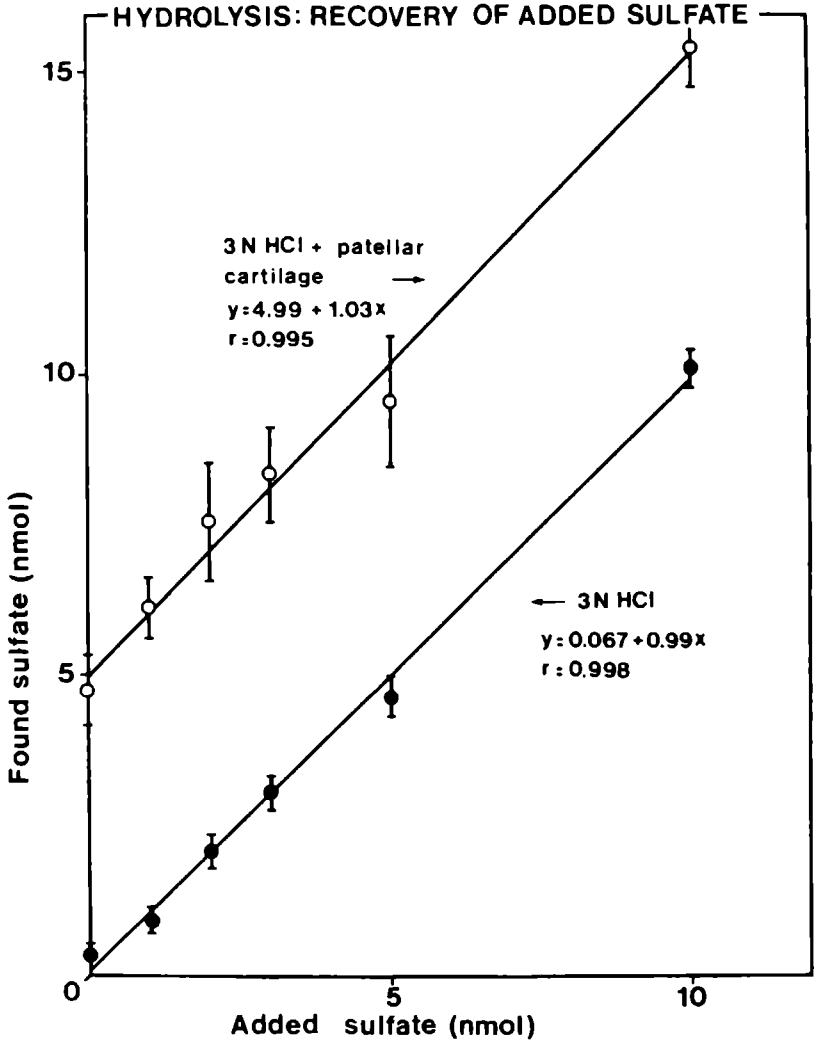

Figure 7. Recovery of SO_4 added to 100 µl 3 M HCL with or without a patellar cartilage layer. Inorganic sulfate analysis was performed, after 24 h at 80°C in closed polypropylene microtubes, on evaporated hydrolysates. Regression lines, calculated from the mean results of 6 sulfate determinations are shown. Correlation is significant. Data represent the mean ± SD of 5 to 6 samples. Coefficient of variance (SD/mean) was always below 15%.

Table 3. Inorganic sulfate liberated from patellar bone and cartilage after various hydrolysis times in 3 M HCl at 80 °C*.

Hydrolysis time (hours)	nmol SO_4 liberated§	
	cartilage	bone
4	3.22 ± 0.77#	0.59 ± 0.30
16	4.80 ± 0.79	0.94 ± 0.48
24	4.49 ± 0.86	0.98 ± 0.68
72	4.66 ± 0.47	1.04 ± 0.51

* Hydrolysis performed in polypropylene Eppendorf tubes.
§ Results are expressed as the mean ± SD of patella specimens obtained from 5 mice.
# $p<0.02$ as compared to the amounts of liberated sulfate from cartilage at later time.

Table 4. Sulfate and GAG contents in cartilage derived from normal, trypsin treated and arthritic patellae.

Patella samples$^{\Delta}$	Contents per cartilage layer*		$(SO_4/GAG)^{o}$
	nmol SO_4 (%)	µg GAG (%)	
Normal patellae	4.58 ± 0.60 (100%)	2.26 ± 0.34 (100%)	(19.5)
Arthritic patellae	3.77 ± 0.52 (82%)$^{\diamond}$	1.70 ± 0.18 (75%)$^{\diamond}$	(21.0)
Trypsin treated patellae	2.71 ± 0.15 (59%)$^{\diamond}$	1.34 ± 0.34 (59%)$^{\diamond}$	(19.4)

Δ See materials and methods section.
* Results are expressed as the mean ± SD of cartilages obtained from 7 mice.
^{o} Percentual ratio calculated on weight basis.
$^{\diamond}$ Values are significantly lower than normal values ($p<0.02$).

mosan induced arthritis, demonstrated equal declines in contents of organic sulfate and cartilage GAGs (20-25 %). These observations indicate that sulfate is firmly associated with the quantitatively removed GAGs. Since the SO_4/GAG ratios seem not affected (Table 4), the GAG sulfation degree in cartilage is probably not significantly changed by trypsin treatment or by inflammation. Finally, experiments done with $^{35}SO_4$-labeled patella revealed that the ^{35}S content in cartilage layers, known

to be fully associated with GAGs, did not significantly differ from the amount of ^{35}S found in benzidine-$^{35}SO_4$ precipitates, obtained from acid hydrolysed cartilages (24 h schedule). Thus there is substantial evidence that virtually all ester sulfate can be released as inorganic sulfate upon acid hydrolysis.

DISCUSSION

Application of microplates instead of cuvettes enables, besides rapid screening, absorbance measurements of small volumes. 100 µl/well is generally sufficient to read the absorbance in a 96-well microplate. With respect to the original benzidine method (14) much smaller sample volumes (10 µl versus 1 ml) can be analysed for inorganic sulfate. Although the sensitivity is not improved (the optical system of a ELISA plate reader is of inferior quality compared to most spectrophotometers), the detection limit is scaled down about 100 times. Nowedays many laboratories possess these ELISA plate multiscans and are familiar with them. So, our version of the benzidine method can easily be introduced in contrast to other existing, very sensitive sulfate assays for which instruments are needed which are often not present or are too expensive to purchase (6,7,9,12).

In our hands the microplate benzidine method is satisfactory. Results correlate well with the established turbidimetric method (2). The sulfate contents can reliably be measured in 10 µl serum, 10 µl diluted urine and in cartilage obtained from one mouse knee cap. Sulfate levels found in mouse and human serum, are in agreement with data reported in several recent publications (9,12,15,19,20). Since only as little as 10 µl mouse serum is needed for analysis, serum sulfate levels in individual mice can be followed in time; blood sampling at the sacrifice of mice, as was formerly done, is not necessary anymore. Influences of factors like drugs, diet, stress (1,2,21,22) on serum sulfate kinetics can now be studied in individual small experimental animals in a relatively easy and rapid manner. As an example sodium salicylate and paracetamol, analgesics commonly used by many people, were demonstrated to decrease serum sulfate in mice. In addition, with our microassay, specific activities of serum ^{35}S-sulfate can be followed, important for in

vivo ^{35}S-sulfate incorporation studies (1). The higher concentration of inorganic sulfate found in mouse urine as compared to that of serum (about 30-50 times) was also observed by others in humans (4,13) and rats (23). Of course, due to these high urine sulfate levels other established macro methods are suitable as well to quantitate urine sulfate in small experimental animals.

Sulfate analysis in human saliva revealed no significant difference in sulfate concentration in comparison with human serum. However, recent studies on saliva sulfate analysis (24) gave another picture; the sulfate levels (0.07 mM) were much lower than in matched serum samples (0.3 mM). Sample collection and preparation of saliva prior to sulfate analysis (by controlled-flow anion chromatography) differed from our method and, perhaps, could explain this discrepancy. Irrespective of medication, serum and synovial fluid of 5 rheumatoid arthritis patients had similar sulfate concentrations, not different from normal serum values.

Reproducable determination of ester sulfate in the range of 0-12 nmol turned out to be impossible when acid hydrolysis is performed in disposable borosilicate glass vials (2 ml). Heating with acid or even with ordinairy water caused release of benzidine precipitating material out of vials purchased from several suppliers, whereas in vials obtained from another supplier sulfate was just absorbing away! These gains or losses in sulfate equivalents were quite variable (2-5 nmol), observed either at 110 °C or 60 °C and even persisted after extensive treatment with chromosulfuric acid followed by exhaustive rinsing with distilled, deionized water. Suchlike problems were also reported by other investigators developing sulfate assays (25,26).

Fortunately, no such complications were encountered with disposable polypropylene Eppendorf tubes (1.5 ml), provided that they were heated not higher than 100 °C. No "sulfate" leakage or absorbance was observed, and recovery of added sulfate was complete (Figure 7). Sixteen hours hydrolysis in 3 M HCl at 80 °C appeared to be sufficient to liberate a maximal amount of inorganic sulfate. Here, also, added sulfate was completely recovered (Figure 7) indicating that this procedure is a reliable one.

Since a considerable amount of SO_4 was detected in hydrolysed patellar bone (up to 20% of the cartilage layer) this part of the patella has to be removed in order to quantitate the cartilage sulfate content. Routinely, chondrocyte metabolic activity is quantitated by radiolabel incorporation using the whole, undamaged patella as a source of anatomically intact cartilage. To this end the patella is removed from the natural surrounding tissue, after formalin fixation and EDTA decalcification (16). However, such a procedure seems not suitable for inorganic sulfate determination, since quantitative removal of the cartilage layer is hardly possible from formalin fixed patellae. Ethanol (96%) fixation followed by decalcification in formic acid (5%) enabled easy and quantitative separation of the cartilage layer from subchondral bone. In this respect it is worthwhile to mention that one should anyway be careful with formalin and EDTA when using the benzidine method, because these agents precipitate benzidine (data not shown).

Ester sulfate determination in cartilage without first isolating proteoglycans or glycosaminoglycans (GAGs) is an unusual procedure. Nevertheless, direct acid hydrolysis of these tiny patellar cartilage specimens (estimated dry weight about 20 µg) turned out to be less cumbersome, and sufficient to liberate all sulfate as, in fact, is indicated by the ^{35}S recovery experiments. Since ^{35}S was shown to be fully associated with sulfated GAGs (1), all ester sulfates are transformed to inorganic sulfate. Evidence for this latter statement is also obtained by the observation that cartilage, derived from trypsin- or arthritic patellae, demonstrates similar decreases in both sulfate content and GAG content, indicating that measured sulfate originated from GAGs. Since the sulfate content is expressed as an amount (nmol) per whole patella (-cartilage) no information is obtained about the GAG sulfation degree. For this to be achieved hexuronate and/or hexosamine contents have to be determined in microgram amounts of purified cartilage GAGs, isolated from mouse patellae. This matter is currently under investigation.

In general, the benzidine method appears to find little present day use. No doubt its known carcinogenicity (27) has contributed to this. However, an analogue of benzidine, 4-amino-4'-chlorodiphenyl (CAD), is

a highly sensitive and selective precipitant for sulfate (6,28) and is reported to be non-carcinogenic (13,29). This compound merits more attention, especially because $(CAD)_2$-SO_4 is less soluble in water then benzidine-SO_4 (28), twice as much CAD is precipitated per sulfate molecule as compared to benzidine, and reported molar extinction coefficients of coloured conjugates of diazotized benzidine and CAD (at 510 nm and 480 nm respectively) are in the same order of magnitude (5,30). From this it is clear that for sulfate determination, benzidine should be replaced by CAD. Unfortunately, as far as we know, CAD is not commercially available at present.

The herein described benzidine method can still be improved and made more easy to perform. Absorbance was measured at 492 nm, since we had an equipment with filters of fixed wavelength, but maximal sensitivity ought to be acchieved at 510 nm (5). To remove all free benzidine after sulfate precipitation, the precipitate is washed once and the tube mouths are wiped with tissue paper. This treatment is a serious drawback in the analytical procedure, since it demands some manipulative skill. Alternatively an extra wash procedure can be introduced with the risk of loosing some precipitate as well. For this purpose a mixture of ethanol-ethyl ether (1:2) is recommended above the ethanol-aceton (1:1) we used (31). Finally, it seems worthwhile to investigate whether the whole assay (precipitation, washing, conjugation, absorbance reading) can be performed in one or two microplates instead of using individual microtubes.

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CHAPTER 4

SALICYLATE-INDUCED DEPLETION OF ENDOGENOUS INORGANIC SULFATE. POTENTIAL ROLE IN THE SUPPRESSION OF SULFATED GLYCOSAMINOGLYCAN SYNTHESIS IN MURINE ARTICULAR CARTILAGE

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SALICYLATE-INDUCED DEPLETION OF ENDOGENOUS INORGANIC SULFATE

Potential Role in the Suppression of Sulfated Glycosaminoglycan Synthesis in Murine Articular Cartilage

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Sodium salicylate has been shown to suppress glycosaminoglycan (GAG) synthesis by articular hyaline cartilage in vitro. We investigated the in vivo effect of sodium salicylate on murine patellar cartilage, using incorporation of intraperitoneally administered ^{35}S-sulfate as a measure of sulfated GAG synthesis. Our results indicated that a single dose of sodium salicylate (200 mg/kg) inhibited in vivo sulfated GAG synthesis by 56%, compared with controls, and had no effect on sulfated GAG breakdown. A striking finding was that sodium sulfate treatment reduced the serum concentration of inorganic sulfate from 1.1 m*M* to approximately 0.3 m*M*, and that this serum reduction was associated with a twofold increase in urinary excretion of sulfate. Using anatomically intact patellar cartilage, in vitro studies clearly showed that, in concentrations reached in vivo (≥1 m*M*), salicylate suppressed murine chondrocyte GAG synthesis. However, in the presence of serum, the effects of 1 m*M* salicylate were abolished. We also found that sulfated GAG synthesis was clearly inhibited at low concentrations of sulfate (<0.5 m*M*). Our data indicate that sodium salicylate can suppress articular chondrocyte sulfated GAG synthesis in vivo, and that this effect may particularly be due to a drug-induced reduction of endogenous sulfate.

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Several nonsteroidal antiinflammatory drugs (NSAIDs) commonly used in the treatment of arthritis have been shown to provoke suppression of glycosaminoglycan (GAG) synthesis in organ cultures of normal articular cartilage (1–3). Studies have clearly shown that salicylate administration suppresses in vitro GAG synthesis in normal human articular cartilage and that degenerated cartilage is affected even more by this drug (4,5). In experiments on canine osteoarthritic cartilage (6), an almost complete suppression was observed at salicylate concentrations of 10 m*M* (14 mg%), a level which is readily achieved in human serum.

Because patients with rheumatic diseases often receive long-term treatment with NSAIDs, it is relevant to know whether the suppressive effect of these drugs, as observed in vitro, also occurs in vivo. A deleterious effect of salicylates on articular cartilage after long-term treatment has been shown in studies of animals (7,8). However, the chondrocyte synthetic function of salicylate-treated animals has only been tested ex vivo (7,9), and a direct action of salicylates on in vivo articular GAG synthesis has not been established.

Originally the present study was aimed at investigations of the short-term effect of sodium salicylate on in vivo sulfated GAG synthesis in normal articular cartilage. A preliminary experiment showed that sodium salicylate indeed inhibited ^{35}S-sulfate incorporation in articular cartilage, but the most striking observation was a decrease in the serum content of ^{35}S-sulfate, which paralleled a decrease in the endogenous serum sulfate level (10). With these observations and those of others (11), who found that the lowering of environmental inorganic sulfate per se may

influence sulfated GAG synthesis by articular chondrocytes, we focused our study on salicylate-induced depletion of endogenous sulfate and its potential effect on in vivo sulfated GAG synthesis. These studies were carried out on anatomically intact murine patellar cartilage (12).

MATERIALS AND METHODS

Animals. We used 7–9 week old male C57BL mice weighing 20–25 gm purchased from the Radiobiological Institute Rijswijk The Netherlands. The mice were housed in plastic cages containing wood shavings fed a commercial pellet diet and given water ad libitum. Mice were in good health showed no signs of infection and had normal weight gain.

In vivo studies. Weight matched mice were injected intraperitoneally with 50–400 mg of sodium salicylate/kg (10 μl/gm). Fifteen minutes after the first injection a group of sodium salicylate–treated mice and a group of saline treated controls received intraperitoneal injections of 2 μCi of $Na_2{}^{35}SO_4$/gm (up to 100 mCi/mmole Radiochemical Centre Amersham England).

Blood and urine samples were taken at various intervals after radiosulfate administration and the ^{35}S content and the concentrations of inorganic sulfate and salicylate were measured. Blood samples were obtained from the tail or by eye extraction at the time of killing. Urine samples were obtained by squeezing the urinary bladder between finger and thumb.

Four hours after ^{35}S sulfate administration mice were killed and whole patellae were dissected from the knee joints in order to measure the amount of ^{35}S sulfate incorporated in the patellar cartilage (see below).

To study the effect of sodium salicylate on in vivo degradation of GAGs, patellae were prelabeled by intraperitoneal injection of radiosulfate (2 μCi of $Na\ ^{35}SO_4$/gm) 48 hours prior to sodium salicylate or saline treatment. Patellae were isolated at the start of the sodium salicylate or saline treatment and 24 hours later. Patellar content of ^{35}S was determined as described below.

Measurement of radioactivity in blood and urine. Blood and urine samples (10 μl) were assayed for radioactive content using 10 ml of Aqualuma plus (Lumac 3M Schaesberg The Netherlands) in a liquid scintillation spectrometer. Blood samples were lysed in 0.5 ml of water before addition of the scintillation fluid.

Measurement of sulfated GAG synthesis. As a measure of in vivo chondrocyte synthetic function the amount of ^{35}S sulfate incorporated in the cartilage of isolated patellae was determined according to the method of van den Berg et al (12, 13). Briefly, patellae with surrounding tissue were dissected from the knee joint, washed with saline, fixed for 1 hour in 4% phosphate buffered formalin (pH 7.0) and decalcified overnight in fixative saturated with Na_2-EDTA. Unbound ^{35}S was removed by thorough washing. The whole anatomically intact patellae were easily punched out from the surrounding tissue after decalcification. Patellae were solubilized in Solulyte (J. T. Baker Chemicals, Deventer The Netherlands) and the incorporated ^{35}S was counted (using Lipofluor scintillation fluid J. T. Baker Chemicals) in a liquid scintillation counter.

To verify that the ^{35}S content of a whole patella may be used as a measure of sulfated GAGs in patellar cartilage we isolated $^{35}SO_4$-GAGs according to the method of Adams and Muir (14). Washed cetylpyridinium chloride–GAG complexes precipitated from papain digests of whole patellae were solubilized in n propanol and the $^{35}SO_4$ GAG content was counted in a liquid scintillation counter as above. More than 90% of the ^{35}S content of whole patellae was recovered in GAGs isolated from them.

Analytic methods. Serum salicylate concentrations were determined by a colorimetric method described by Greiling and Schuler (15). Salicylate standards were established using normal mouse serum obtained from age matched mice. By using microplates (instead of cuvettes) and a Titertek Multiscan (Flow Laboratories Irvine Scotland) to read the absorbance 20 μl of serum was sufficient for a duplicate determination (reproducible within a range of ±10%).

Inorganic sulfate was measured by a turbidimetric method that has been described and modified by Knigsheld et al (16, 17). To 500 μl of (diluted) serum or urine containing 0.1–1.3 m*M* inorganic sulfate 2 ml of trichloroacetic acid solution (5% weight/volume) was added and the mixture was allowed to stand at room temperature for 10 minutes. After centrifugation 1 ml of clear supernatant was added to 250 μl of $BaCl_2$ reagent (20 gm of $BaCl_2 2H_2O$ and 100 gm of dextran in 1 liter of distilled water) in a disposable semi microcuvette and after 35 minutes the absorbance (at 360 nm) was read against a sample background (1 ml of supernatant and 250 μl of reagent containing 100 gm of dextran liter of distilled water).

To determine the percentage of the inorganic form ($^{35}SO_4$) of ^{35}S in the serum samples from radiosulfate-treated mice were analyzed as follows. After determination of the serum sulfate concentration by the turbidimetric method the contents of the cuvettes were centrifuged and the pellet (inorganic sulfate fraction in the form of precipitable $Ba^{35}SO_4$) and the supernatant were counted. In normal mice 97% of ^{35}S was in the inorganic form ($^{35}SO_4$) in sodium salicylate–treated mice it was slightly less but was always >90% of the total serum ^{35}S content. In both the treated and the untreated mice the ^{35}S content in blood paralleled that in the derived serum. Thus blood samples could be used as equivalents of serum samples in measurements of inorganic radiosulfate content.

In vitro studies. Normal male C57BL mice were killed by cervical dislocation and patella specimens were carefully removed from the knee joints. The patellae were incubated as described (12) using RPMI culture medium (200 μl/patella) containing gentamycin (50 μg/ml) L glutamine (2 m*M*) and Na_2SO_4 (0.4 m*M*). Sodium salicylate was added in concentrations ranging from 1×10^{-6} *M* to 5×10^{-1} *M* medium without sodium salicylate served as the control.

After 2 hours at 37°C under a 5% CO_2 atmosphere the patellae were transferred to fresh medium containing sodium salicylate in the original concentrations. The sulfate concentration was increased to 0.5–0.6 m*M* by addition of 10 μCi of $Na_2{}^{35}SO_4$/ml and incubation was continued for an

additional 2 hours. Patellae were then processed for ^{35}S counting as described above.

To investigate the effects of low sulfate concentration on chondrocyte GAG synthesis, a sulfate depleted medium was used. Patellae with surrounding tissue were incubated in basal minimal Eagle's (BME) diploid medium (Flow Laboratories) with added gentamycin (50 μg/ml) in which the amount of inorganic sulfate was adjusted with Na_2SO_4 to give the desired concentration (0.1–1.3 m*M*). After 1 hour of preincubation, the patella specimens were transferred to $Na_2{}^{35}SO_4$ containing BME diploid medium (10 μCi/ml) with SO_4^{--} concentrations the same as those used during preincubation, and incubation was continued for an additional 2 hours.

The amount of incorporated sulfate (in mmoles) was calculated from the ^{35}S-sulfate content (in disintegrations per minute) of the patellar cartilage and the specific activity of $^{35}SO_4^{--}$ in the incubation medium (dpm/mmole).

Statistical analysis. Statistical significance was calculated using Student's *t* test and analysis of variance.

RESULTS

Effect of sodium salicylate on in vivo rate of ^{35}S-sulfate incorporation in patellar cartilage. Thirty minutes after a single intraperitoneal injection of 200 mg of sodium salicylate/kg, peak serum concentration of salicylate (2.8×10^{-3} *M*, 38 mg%) was reached (Figure 1). At 4 hours postinjection, the concentration was still $>10^{-3}$ *M* (14 mg%), this is a concentration which may inhibit in vitro GAG synthesis (see below). Mice that were treated with this 200 mg/kg dose received an intraperitoneal injection of $Na_2{}^{35}SO_4$ 15 minutes later and patellae were isolated 4 hours thereafter.

The amount of ^{35}S-sulfate incorporated in the patellae of sodium salicylate–treated mice was reduced to 56 ± 17% (mean ± SD) ($P < 0.001$), compared with saline treated controls (Table 1). Similar inhibition values were obtained when isolated GAGs were counted instead of whole patellae, indicating that the decreased ^{35}S-sulfate incorporation rate signifies a suppressed sulfated GAG synthesis.

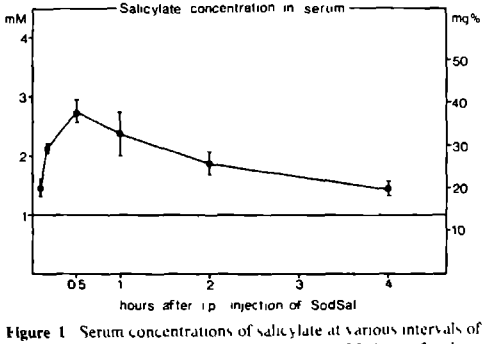

Figure 1. Serum concentrations of salicylate at various intervals of time after intraperitoneal (i.p.) administration of 200 mg of sodium salicylate (SodSal)/kg. Each dot represents the mean ± SD for 6 mice. Salicylate concentrations above the shaded area demonstrate a suppressive effect on in vitro articular glycosaminoglycan synthesis (see Table 3).

Table 1. Measurement of in vivo incorporation of ^{35}S into patellar cartilage and blood content of ^{35}S after sodium salicylate or saline (controls) treatment*

	^{35}S content		
	Whole patellae (cpm)	Isolated GAGs (cpm)	Blood (cpm × 10^3/10 μl)
Control	190 ± 28	175 ± 30	6.6 ± 0.8
Sodium salicylate	107 ± 29 (56)†	102 ± 19 (58)	2.0 ± 0.3 (30)†

* Values represent the mean ± SD for 6 mice. GAGs = glycosaminoglycans. Numbers in parentheses are % inhibition compared with controls. See Materials and Methods for details.
† Significantly different from controls, $P < 0.001$ by Student's unpaired 2 tailed *t* test.

To examine the effect of sodium salicylate on in vivo degradation of GAGs, patellae were prelabeled by intraperitoneal injection of radiosulfate 48 hours before sodium salicylate or saline treatment. The ^{35}S content in prelabeled patellae of control mice was significantly decreased 24 hours after saline treatment (Table 2), indicating that a measurable $^{35}SO_4$ GAG degradation had occurred. Sodium salicylate treatment seemed to have no effect on this in vivo breakdown. A surprising observation in the mice given ^{35}S-sulfate shortly after sodium salicylate administration was the low content of ^{35}S in their blood at the time of killing (Table 1); this could also be the reason for the decreased rate of ^{35}S-sulfate incorporation in patellar cartilage.

Effect of sodium salicylate on the ^{35}S content in blood. The blood content of ^{35}S was already signifi-

Table 2. In vivo effect of sodium salicylate treatment on the ^{35}S content of prelabeled patellae*

Group	Treatment	Time (hours)	^{35}S content cpm/patella (%)
1	–	0	184 ± 31 (100)
2	Saline	24	128 ± 11 (70)
3	Sodium salicylate	24	131 ± 27 (71)

* Values represent the mean ± SD for 6 mice. Patellae were isolated from group 1 mice 48 hours after intraperitoneal administration of 2 μCi of $Na_2{}^{35}SO_4$/gm; groups 2 and 3 mice were treated with intraperitoneal injections of saline and sodium salicylate (200 mg/kg), respectively, and their patellae were isolated 24 hours later.
† Significantly different from group 1, $P < 0.001$ by Student's unpaired 1 tailed *t* test. There was no significant difference between groups 2 and 3.

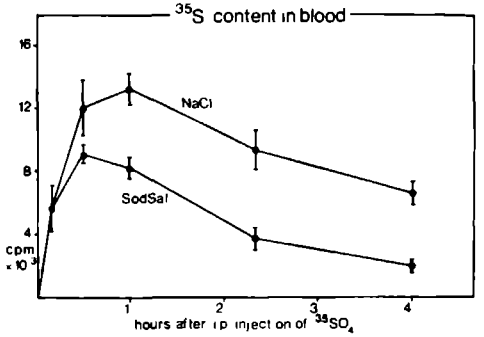

Figure 2 Effect of sodium salicylate (SodSal) on the blood content of ^{35}S. Fifteen minutes after intraperitoneal (i.p.) administration of either 700 mg of sodium salicylate/kg or saline, mice received intraperitoneal Na $^{35}SO_4$. Each dot represents the mean + SD for 6 mice.

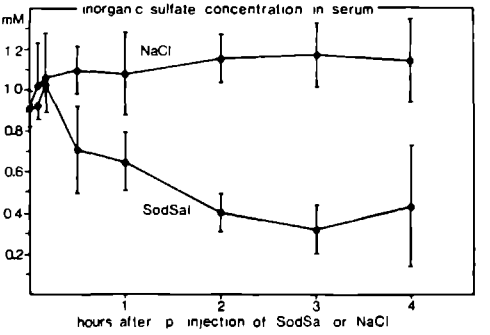

Figure 3 Effect of intraperitoneal (i.p.) sodium salicylate (SodSal) (200 mg/kg) administration on the concentration of inorganic serum sulfate. Control mice received saline. Each dot represents the mean ± SD for 5 mice.

cantly decreased in the early phase after sodium salicylate treatment (200 mg/kg) and this difference between the sodium salicylate–treated group and the control group gradually increased thereafter (Figure 2). This phenomenon was accompanied by an increased urinary excretion of ^{35}S which reached 200% of control values at approximately 45 minutes. Two hours after sodium salicylate administration urinary ^{35}S excretion was almost the same as that in the control group and at 22 hours ^{35}S content ultimately declined to 50% of control values.

Effect of sodium salicylate on the endogenous inorganic sulfate concentration in serum. As was found for ^{35}S, sodium salicylate administration (200 mg/kg) caused a marked decrease in the endogenous sulfate concentration in serum. This lasted for at least 3 hours. The concentration of inorganic sulfate decreased from the physiologic serum level of 1.1 m*M* to approximately 0.3 m*M* (Figure 3). The decrease in serum concentration was accompanied by increased urinary excretion of inorganic sulfate within 2 hours after drug treatment.

The decline in serum concentration of SO_4^{--} (in m*M*) coincided with the decrease in serum content of ^{35}S (in counts per minute). Consequently, the specific activity of circulating ^{35}S-sulfate is virtually the same in sodium salicylate–treated and control mice (Figure 4). This indicates that the decreased ^{35}S-sulfate incorporation found in the patellar cartilage (Table 1) in fact represents a suppressed rate of incorporation of inorganic sulfate.

Table 3 shows the dose dependency of the sodium salicylate–induced decrease in inorganic serum sulfate levels. We found that a 50-mg/kg dose induced a significant effect on serum content of sulfate.

In vitro effects of sodium salicylate and low sulfate concentration on the rate of sulfate incorporation in patellar cartilage. With the data obtained thus far it was still unclear whether sodium salicylate itself caused decreased sulfate incorporation by acting directly on the chondrocyte or whether the sulfate drop (caused by sodium salicylate) was responsible for this phenomenon. To investigate these aspects separately we conducted in vitro experiments.

The direct effect of sodium salicylate was studied by incubating whole patellae for 3 hours in sodium

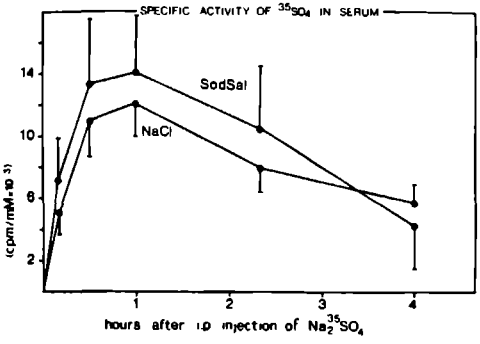

Figure 4 The specific activity of inorganic radiosulfate in the serum of mice treated intraperitoneally (i.p.) with sodium salicylate (SodSal) and with saline. Values are derived from the data shown in Figures 2 and 3.

Table 3. Serum salicylate and sulfate concentrations 2 hours after intraperitoneal treatment with sodium salicylate*

Dose (mg/kg)	Serum salicylate (m*M*)	Serum sulfate (m*M*)
0	0	1.01 ± 0.05
50	0.5 ± 0.2	0.79 ± 0.14
100	1.1 ± 0.1	0.58 ± 0.11
400	3.1 ± 0.4	0.47 ± 0.08

* Values represent the mean ± SD for 3 mice. There was a significant sodium salicylate–mediated decrease in serum sulfate concentration, $P < 0.05$ by one-way analysis of variance.

salicylate–containing medium, the last hour in the presence of radiosulfate. As measured by ^{35}S incorporation, sodium salicylate exerted a dose-dependent suppression on the incorporation of sulfate into sulfated GAGs of patellar cartilage (Table 4). However, the suppressive effect of 10 m*M* sodium salicylate was completely abolished when the culture medium was supplemented with ≥25% normal mouse serum.

The effect of a reduced sulfate concentration on patellar chondrocyte metabolism was examined by incubating whole patellae in medium with various concentrations of sulfate (0.0–1.2 m*M*). At serum concentrations of sulfate varying about the range of the normal endogenous level (1.0 m*M*), the rate of sulfate incorporation was hardly affected. However, sulfate incorporation in patellar cartilage was found to be decreased at sulfate concentrations below 0.5 m*M* (Figure 5), such concentrations are readily achieved after in vivo sodium salicylate treatment.

DISCUSSION

Our data indicate that treatment of mice with salicylate caused a clear reduction of in vivo sulfate incorporation in articular cartilage and a marked fall in the endogenous serum sulfate level. In addition to a direct action of salicylate on the chondrocyte, the drug-induced decrease in sulfate availability may be a potential mechanism for the reduced sulfate incorporation, since under low sulfate concentrations in vitro, sulfated GAG synthesis was shown to be highly suppressed.

Table 4. In vitro effect of sodium salicylate on ^{35}S incorporation in anatomically intact patellar cartilage*

Sodium salicylate concentration (moles/liter)	*Incorporated* ^{35}S (% of control)	*P*†
5×10^{-3}	54 ± 11	<0.001
1×10^{-3}	81 ± 11	<0.01
1×10^{-5}	110 ± 13	NS
1×10^{-6}	109 ± 19	NS

* Values represent the mean ± SD for 5 mice. Patellae were incubated for 3 hours, for the last hour in the presence of radiosulfate. Control patellae were cultured in the absence of salicylate.
† Versus controls. Calculated using Student's unpaired 2-tailed *t*-test. NS = not significant.

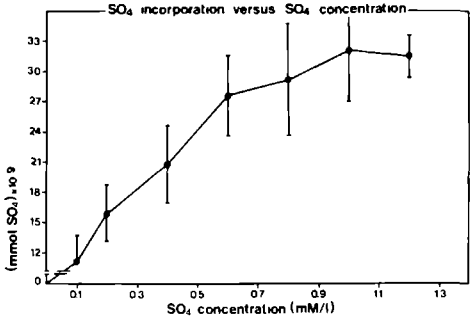

Figure 5. Sulfate incorporation into sulfated glycosaminoglycans by normal murine patellar cartilage cultured in the presence of various concentrations of sulfate. Results are expressed as mmoles of sulfate incorporated into the cartilage of 1 patella in 2 hours. Each dot represents the mean ± SD for 5 mice.

After a single intraperitoneal dose of sodium salicylate, 200 mg/kg, a serum salicylate concentration between 15–40 mg% (1–3 m*M*) was reached in these mice, and this treatment was found to induce a significant reduction in the ^{35}S incorporation in the articular cartilage in vivo. Similar results (with salicylate and other drugs) have been reported for a number of tissues (18–21), and these data have been interpreted as demonstrating a drug-induced inhibition of sulfate incorporation and therefore a reduced sulfated GAG synthesis. However, our analysis of serum sulfate levels after salicylate treatment showed a rapid fall in the endogenous sulfate level, hence, changes may occur in the specific activity of the ^{35}S-sulfate in the drug-treated mice, and these changes are dependent on the timing of the radiolabel administration. Therefore, exact knowledge of the specific activity is an absolute prerequisite for translating ^{35}S incorporation values into chondrocyte sulfated GAG synthesis.

The best way to examine drug-induced changes in sulfate levels is to examine blood samples, although Herbai (21) described an elegant double isotope method of determining endogenous sulfate levels from 1 droplet of urine. Analysis of 24-hour urine samples, the method used by Bostrom et al (22) to measure a drug's effect on the endogenous sulfate concentration, seems unsuitable since temporary increases and decreases in urinary sulfate excretion after drug treatment, as observed in our experiment, escape notice in such an

overall measurement In the experimental method we used, the specific activity of ^{35}S-sulfate remained at the same level in salicylate-treated and control mice, therefore, the decreased ^{35}S content of the patellar cartilage could be interpreted as a decrease in the amount of newly formed sulfated GAGs This must be due to inhibited synthesis, since salicylate was shown to have no effect on the in vivo degradation of prelabeled GAGs from the patellar cartilage (Table 2)

Studies were performed with only ^{35}S-sulfate as a radiolabel, this gave information about the sulfation of GAGs and not about the synthesis of the carbon chain of GAGs Although ^{35}S-sulfate incorporation is commonly used as a measure of GAG synthesis, some care must be taken in drawing general conclusions, especially since sulfation and GAG backbone synthesis can occur uncoupled.

A distinct decline in the endogenous sulfate level was observed after a 50-mg/kg dose of sodium salicylate It seems unlikely that drug sulfation is responsible for this decrease in serum sulfate since neither salicylic acid nor its sodium salt or acetyl derivative is a substrate for sulfation (15,23,24). Furthermore, chemically sulfated salicylic acid is completely hydrolyzed in an aqueous environment (15) The most reliable explanation is a salicylate induced diuresis occurring by direct action on the kidneys, since levels of inorganic sulfate excreted in the urine were increased twofold shortly after drug treatment.

There are at least 2 potential mechanisms for the salicylate-mediated suppression of in vivo sulfate incorporation into the articular cartilage First, salicylate could exert a direct effect on in vivo chondrocyte GAG synthesis, as it does in vitro Second, the decreased endogenous sulfate level could be the causative factor.

Palmoski and Brandt (25) showed that salicylate concentrations in excess of 10 ^{3}M caused a clear inhibition of chondrocyte GAG synthesis in canine cartilage in vitro, measured either by ^{35}S-sulfate or ^{3}H-glucosamine incorporation It is believed that salicylate provokes suppression of GAG synthesis by decreasing the availability of glucuronic acid, one of the major components of GAGs. This is caused either by the inhibition of the activity of uridine diphosphate (UDP)–glucose dehydrogenase, which catalyzes the conversion of UDP-glucose to UDP–glucuronic acid (25,26), or by conjugation of glucuronic acid to salicylate itself (19). Our in vitro studies made it clear that salicylate may also exert an inhibition of chondrocyte GAG synthesis in intact murine articular cartilage However, in the presence of 25% mouse serum, the inhibitory effect of $10^{-3}M$ salicylate was abolished, and this casts some doubt on the relevance of this mechanism in vivo This may be due to protein binding of salicylate, resulting in a decreased concentration of free salicylate

Regarding the second mechanism, lowered sulfate availability as a factor causing inhibition of sulfated GAG synthesis in vivo, we have obtained suggestive in vitro evidence Maximal sulfate incorporation was seen at approximately 1 mM sulfate (the physiologic sulfate level in the serum of C57BL mice) (27), and a distinct decrease in sulfate incorporation was demonstrated at sulfate concentrations below 0 5 mM (Figure 5), which are readily achieved in vivo after salicylate treatment (Figure 3) The dependence of sulfate incorporation on the sulfate concentration has been described by Maroudas and Evans (11) in their studies of human articular cartilage They concluded that inorganic sulfate must be one of the rate-limiting substrates in the synthesis of sulfated GAGs Maximal sulfate incorporation was found at approximately 0.4 mM sulfate, the concentration that is physiologic for humans (28), and a clear decrease was found at concentrations below 0 2 mM.

The observation of a plateau level of sulfate incorporation occurring with sulfate concentrations varying widely about the physiologic range in each of the 2 species may indicate the existence of an important homeostatic mechanism. Thus, the clearly observed fluctuations in endogenous sulfate levels corresponding with circadian rhythm (28) have no implications for cartilage metabolism.

Besides penetration of various solutes, the resilient and load-bearing properties of articular cartilage are closely dependent on the concentration of negatively charged ionic groups Of these, only sulfate groups (–OSO$_3^-$) are responsible for a stable interaction of proteoglycans with collagen, the 2 major matrix components of cartilage (29) Hence, drug-produced inhibition of chondrocyte sulfated GAG synthesis may cause a loss of cartilage integrity Wilhelmi's finding that acetylsalicylic acid, administered once daily for 5 months in oral doses of 50 mg/kg and 150 mg/kg, promoted the development of spontaneous osteoarthritis in C57BL mice (8) is relevant in this respect

Further evidence for in vivo drug-induced cartilage damage emerged from studies by Palmoski and Brandt (7), who found that experimental canine osteoarthritis was aggravated in animals that received daily doses of aspirin for 9 weeks Interestingly, this dose regimen had no apparent effect on the cartilage of the contralateral normal joint, indicating that arthritic car-

tilage may be more vulnerable to salicylate-induced damage

Finally since the kinetics of pharmacologic response to salicylates are likely to be different between animal species as well as between animals and humans, one should be cautious in interpreting these data for diseases in humans

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CHAPTER 5

THE EFFECT OF SALICYLATE ON ANATOMICALLY INTACT ARTICULAR CARTILAGE IS INFLUENCED BY SULFATE AND SERUM IN THE CULTURE MEDIUM

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The Effect of Salicylate on Anatomically Intact Articular Cartilage is Influenced by Sulfate and Serum in the Culture Medium

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Abstract **The established, suppressive effect of salicylate on sulfated glycosaminoglycan (sGAG) synthesis by normal articular cartilage was reinvestigated using anatomically intact articular cartilage of the whole mouse patella. Employing the physiological murine sulfate concentration (1.0 mM) sodium salicylate (1–5 × 10^{-3} M) caused a dose dependent inhibition of ^{35}S-sGAG synthesis (10–35%). At a lower sulfate concentration (0.4 mM) this inhibition was increased (15–45%) and the suppression was even more pronounced in sulfate deprived medium. This observation stresses the need of using physiological sulfate concentrations in cartilage culture studies. In the presence of 100% serum the therapeutic drug concentration (1–2 × 10^{-3} M) had no longer any suppressive effect, either at 1.0 mM or at any lower sulfate concentration. Our data suggest that salicylate has no *direct* effect on sGAG metabolism in normal articular cartilage *in vivo* and that adverse effects may be due to the observed salicylate induced lowering of the endogenous sulfate level. (*J Rheumatol 1986,* 13:686–693)**

Key Indexing Terms
ARTICULAR CARTILAGE
SODIUM SALICYLATE
SERUM
SULFATE CONCENTRATION
GLYCOSAMINOGLYCAN SYNTHESIS

Salicylates and several other nonsteroidal antiinflammatory drugs (NSAID) have been shown to inhibit glycosaminoglycan (GAG) synthesis by articular cartilage *in vitro*[1-3] The initial studies were mainly performed with cartilage slices which contained damaged artificial surfaces In a recent paper it has been suggested that the proteoglycan content of the cartilage matrix may be an important determinant of the susceptibility of articular cartilage to drugs[4] It was found that the chondrocyte GAG synthesis was more suppressed when the proteoglycan content of normal cartilage had been reduced by hyaluronidase digestion before incubation with the drug The higher vulnerability to salicylate observed in osteoarthritic (OA) cartilage[5] as well as in articular cartilage from unloaded regions[4] may also be related to the decreased proteoglycan content of these tissues

Since it has been noted that significant proteoglycan loss may occur upon culture of cartilage slices close to the newly cut surfaces[6,7], drug studies should preferably be performed with intact cartilage, exposing only the articular surface to the culture medium In our study we examined in detail the *in vitro* effect of salicylate on chondrocyte GAG synthesis by anatomically intact patellar cartilage of the mouse To resemble the *in vivo* condition, special attention was given to the influence of serum in the incubation medium Since we have recently shown that the suppressive effect of sodium salicylate, as observed *in vivo*, is probably not due to a direct effect of the drug on chondrocyte synthesis, but to a salicylate induced sulfate diuresis, which resulted in a reduced availability of endogenous, inorganic sulfate[8], we further examined the influence of variations in the inorganic sulfate concentration.

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MATERIALS AND METHODS

Animals Male C57Bl mice 6–10 weeks of age and weighing 20–27 g were used They were matched for weight (mean ± 1 0 g) and age (mean ± 0 5 week) before the start of each experiment

Cartilage culture Patellar cartilage was used as a source of articular cartilage GAG metabolism was studied by described methods[9,10] In brief, mice were sacrificed by cervical dislocation and both intact patellae with surrounding tissue still attached were dissected carefully For each test freshly isolated patella specimens from groups of 5 to 6 mice were incubated in medium consisting of RPMI (200 µl/patella), L glutamine (2 mM) gentamycin (50 µg/ml) and 0 4 mM SO_4^{2-} (all from Flow Laboratories, Irvine, Scotland) In the presence of various concentrations of sodium salicylate (SodSal) all incubations were performed at 37°C under a 5% CO_2 atmosphere for short term periods, most of them lasting no longer than 4 h For the final 2 h whole patellae were radiolabeled with D-[6-^{3}H] glucosamine HCl (20 µCi/ml) or ^{35}S-sulfate (Radiochemical Centre, Amersham, England) to assess GAG synthesis Both carrier and carrier-free ^{35}S sulfate were used (10–40 µCi/ml), the former raised the sulfate concentration in the RPMI medium from 0 4 mM to 0 5-0 7 mM SO_4 GAG breakdown was studied by pulsing patellae with ^{35}S sulfate for 2 h before exposure to salicylate The amount of radiotracer incorporated with-

in this period was accepted as 100% ^{35}S GAG breakdown rate was examined over the next 6 h culture with and without SodSal

Determination of radiolabeled GAG ^{35}S sulfate and ^{3}H glucosamine incorporation into the patellar cartilage GAG were determined in a manner previously described[8,9] and briefly outlined here Radiolabeled patellae with surrounding tissue were fixed in 4% phosphate buffered formaldehyde (pH 7 0) and decalcified overnight in Na_2EDTA saturated fixative Nonincorporated free label was removed by thorough washing Due to decalcification the whole anatomically intact patellae could easily be isolated from their surrounding tissue ^{35}S /^{3}H GAG were extracted from patellae by means of papain digestion (200 μl/patella) followed by cetylpyridinium chloride precipitation as described[11] Carrier GAG (50 μg/100 μl) consisting of chondroitin sulfate 4 and 6 and hyaluronic acid (3 3 1) were present in the papain digestion medium to obtain adequate precipitation of the radiolabeled GAG After solubilization in Solulyte (J T Baker Chemicals Deventer NL) the ^{35}S and ^{3}H contents in patellar GAG were counted by means of the scintillation fluid Lipofluor (J T Baker as above) in a liquid scintillation counter (^{3}H window 0 4–8 keV ^{35}S window 15–160 keV) The results were expressed as counts/min (CPM) Also the ^{35}S and ^{3}H contents in whole patellae were determined by direct solubilization in Solulyte instead of first isolating GAG

Sulfate depletion of serum Normal mouse serum obtained from mice 8 weeks of age was depleted from SO_4 with $BaCl_2$ $BaSO_4$ precipitate was removed by centrifugation at 10 000 RPM for 30 min Care was taken not to use an excess of Ba^{++} over $SO_4^{=}$ to avoid undesirable side effects of Ba^{++} This condition was always met by exact determination of the sulfate concentration and then adding Ba^{++} to serum $SO_4^{=}$ in the ratio 1 1 The sulfate concentration was decreased from about 1 0 mM to below 0 1 mM but was still detectable with our sulfate assay (see below) indicating that no free Ba^{++} was present

Ex vivo study Groups of mice were injected intraperitoneally with 200 mg SodSal/10 ml water/kg or 10 ml physiological saline/kg (controls) After blood sampling via heart puncture mice were sacrificed at different times ½ 1 2 3 24 h) Immediately after dissecting patella specimens radiolabeling was performed in ^{35}S sulfate containing RPMI medium for one h Patellar ^{35}S content was assessed as described above

Analytical methods Inorganic sulfate was determined by a modification of the benzidine method described by Spencer *et al*[12] Serum salicylate was assayed by a modification of the method used by Greiling and Schuler[13]

Statistical analysis Differences between means were considered statistically significant when $p < 0\ 05$ according to Student s t test for unpaired data

RESULTS

Effect of SodSal on patellar GAG synthesis Whole patellae with intact articular cartilage were cultured in RPMI medium in the presence of various SodSal concentrations Chondrocyte GAG synthesis was assessed with ^{35}S-sulfate and ^{3}H glucosamine incorporation To verify the necessity of isolating GAG for quantitating newly formed GAG, the ^{35}S and ^{3}H content of whole patellae as well as of isolated patellar GAG were determined As depicted in Table 1, the ^{35}S content of whole patellae and of GAG quantitatively isolated from them are almost the same, as are the inhibition values provoked by SodSal The amount of ^{3}H-glucosamine incorporated in whole patellae is, however, much higher compared with isolated GAG, indicating that ^{3}H glucosamine is also incorporated into non-GAG compounds This makes clear that for radioglucosamine, GAG isolation is an absolute prerequisite Both ^{3}H and ^{35}S incorporation into GAG are depressed by salicylate, indicating inhibition of the whole GAG backbone and not merely sulfation

In further experiments only ^{35}S sulfate incorporation was used because of the ease of assessing sulfated GAG (sGAG) synthesis simply counting the amount of ^{35}S incorporated in the whole patella Using radiosulfate we noticed that chondrocyte sGAG synthesis was only moderately inhibited by therapeutic concentrations of salicylate ($1-2\times10^{-3}$M) It is of interest that low SodSal concentrations tend to stimulate sGAG synthesis (Table 1) Although the effect seldom reaches statistical significance, this phenomenon has repeatedly been observed

Table 2 shows the effect of SodSal on the normal release of ^{35}S GAG from prelabeled patellae After an incubation period of 6 h in drug free medium, the ^{35}S content was significantly decreased ($p<0\ 02$), indicating that a measurable release of ^{35}S-GAG took place Even at high concentrations of SodSal no adverse effect was demonstrat-

Table 1 *Effects of SodSal on the incorporation rate of ^{35}S-sulfate and ^{3}H glucosamine in whole patellae and isolated glycosaminoglycans**

SodSal	^{35}S Content‖				^{3}H Content‖			
	CPM ± SD (% of control)				CPM ± SD (% of control)			
(Mol/l)	Whole Patellae	p†	Isolated GAG	p	Whole Patellae	p	Isolated GAG	p
Control	415 ± 60 (100)		396 ± 67 (100)		1735 ± 275 (100)		1130 ± 103 (100)	
1×10^{-2}	178 ± 30 (43)	<0 001	162 ± 19 (41)	<0 001	1492 ± 134 (86)	NS	746 ± 82 (66)	<0 001
5×10^{-3}	270 ± 59 (65)	<0 01	249 ± 32 (63)	<0 001	1579 ± 208 (91)	NS	802 ± 136 (71)	<0 001
2×10^{-3}	340 ± 38 (82)	<0 04	—	—	—		—	—
1×10^{-3}	374 ± 64 (90)	NS	—	—	—	—	—	—
1×10^{-4}	461 ± 69 (111)	NS	—	—	—	—	—	—
1×10^{-5}	457 ± 91 (110)	NS	—	—	—		—	—

* 4 h s incubation final 2 h with both radiotracers

‖ Values are mean ± SD of patellae from groups of 6 mice

† Statistical significance of difference from control (unpaired 2 tailed) NS=not significant

Table 2 *Effect of SodSal on the normal release of ^{35}S-GAG from prelabeled patellae*

Group	SodSal (Mol/l)	Time (h)	^{35}S Content[§] (CPM/Patella)	(%)
1	—	0	712 ± 63	100
2	—	6	583 ± 90	82[¶]
3	2×10^{-3}	6	586 ± 83	82
4	5×10^{-3}	6	527 ± 34	74
5	1×10^{-2}	6	574 ± 87	80

§ Patellar ^{35}S contents represent mean ± SD of patellae from groups of 6 mice

¶ Group 2 versus group 1 $p<0.02$, unpaired 2 tailed Student s t test No significant difference between Group 2 and SodSal treated groups[3 5]

ed on this catabolic process Thus the drug mediated decreased ^{35}S content in patellar cartilage, as listed in Table 1, is solely due to a salicylate provoked suppression of sGAG synthesis and not to accelerated release of preformed ^{35}S-GAG

SodSal effect time dependence To examine the time dependence of the suppressive effect of salicylate on GAG synthesis, patellae were preincubated with 2×10^{-3}M SodSal for various periods, ranging from 0 to 5 h Subsequently, radiosulfate labeling was performed in drug containing medium for one h Twenty percent inhibition of ^{35}S incorporation was already found without preincubation and this effect was not aggravated by prolongation of the drug exposure time

SodSal effect reversibility The ability of patellar chondrocytes to recover from the suppressive effect of salicylate was studied by preincubating patellae for 3 h in a rather high concentration of SodSal (10^{-2}M), followed by a variable chase period in excess medium (1 2 ml/patella) without drug ^{35}S labeling was performed for only 30 min immediately after chase incubation Figure 1 shows a gradual recovery of the suppressed ^{35}S incorporation rate with increasing chase time, after 2 h control values were already reached

SodSal effect age dependence Young and aged articular cartilage, assumed to be different in chondrocyte function as well as in matrix composition, were assessed for the suppressive action of salicylate Whole patellae of mice (8,15,27-weeks-of-age) were cultured during 4 h in the presence of 5×10^{-3}M SodSal, the final 2 h with ^{35}S-sulfate Although the control ^{35}S incorporation rate in whole patellae clearly diminished with increasing age, the SodSal mediated suppression remained identical (Table 3)

SodSal effect influence of sulfate concentration In previous studies it has been shown that sodium salicylate, administered *in vivo*, provoked a drastic decrease of the endogenous inorganic sulfate level[8] To investigate possible interference of environmental sulfate with the suppressive action of salicylate, patellae were incubated with SodSal at 2 different sulfate concentrations The normal serum level in C57Bl mice (1 0 mM SO_4^{2-}) and the level reached after *in vivo* SodSal (200 mg/kg) treatment (0 4 mM SO_4^{2-}) were applied ^{35}S labeling was done with carrier free ^{35}S-sulfate

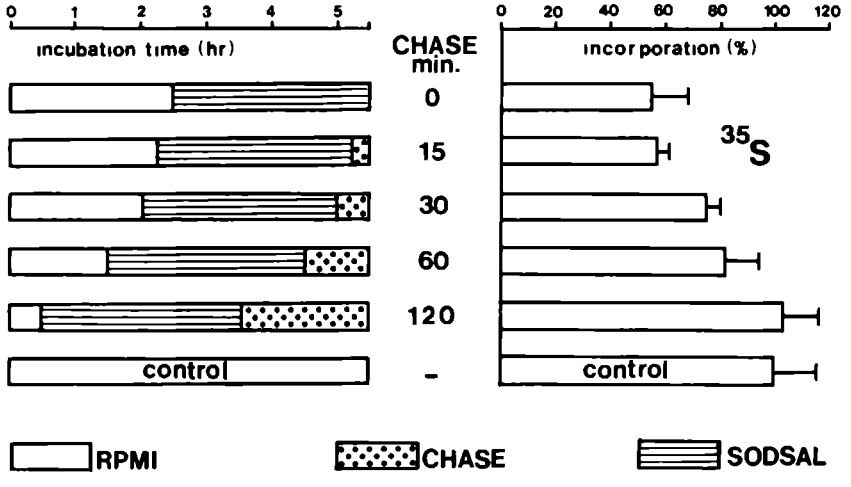

Fig 1 Reversibility of the SodSal (10^{-3} M) mediated inhibition of ^{35}S incorporation in whole patellae ^{35}S labeling was performed for 30 min immediately after chase incubation Incorporation values (% of control) represent mean ± SD of patellae from groups of 6 mice After a 2 h chase period the difference from control is no longer statistically significant (unpaired and one tailed Student s t test)

Table 3 *Effect of SodSal (5×10 ⁻³M) on the ³⁵S incorporation rate in whole patellae age dependence*

Age	CPM patella[¶]		
	Control	SodSal	% of Control[§]
8 weeks	1069 ± 207	578 ± 49[†]	54
15 weeks	678 ± 77	332 ± 31	49
27 weeks	574 ± 92	294 ± 55[†]	51

¶ Data represent mean ± SD of patellae from groups of 6 mice
† Significantly different from controls $p<0.001$ (unpaired 2 tailed)
§ No significant difference in the SodSal mediated suppression between the various age groups

which had a negligible contribution to the sulfate concentration in the culture medium A tendency of a more pronounced inhibition of ^{35}S incorporation at the lower sulfate concentration was often found (Table 4) The therapeutic SodSal concentration of the 10^{-3}M affected chondrocyte sGAG synthesis only at a low inorganic sulfate level Many ^{35}S labeling studies are performed in medium without sulfate to obtain a high ^{35}S incorporation rate Under such conditions drug effects may be artifactually overestimated (Table 5)

SodSal effect influence of serum To examine physiological concentrations of free salicylate in the presence of protein bound salicylate, incubation experiments were performed in

Table 4 *SodSal effect on ³⁵S incorporation in whole patellae at 2 sulfate concentrations*

SodSal (Mol l)	^{35}S content (%)[¶]		p[†]
	1 0 mM SO_4	0 4 mM $SO_4^=$	
1×10^{-3}	92 ± 7 (n=9)	84 ± 6 (n=6)	<0 05
2×10^{-3}	84 ± 7 (n=10)	74 ± 6 (n=5)	<0 02
5×10^{-3}	64 ± 8 (n−5)	56 ± 8 (n=5)	NS

¶ Values are expressed as the mean ± SD of the average percentages ^{35}S incorporated in drug treated versus control patellae in separate experiments (n) Each experiment consisted of patellae obtained from groups of 5 to 6 mice
† Significance of difference between the values obtained at 2 sulfate concentrations (unpaired 2 tailed test) NS – not significant

100% serum which shares many properties with synovial fluid (SF) In the therapeutic range ($1\text{-}2 \times 10^{-3}$M) either mouse or human serum totally abolished the suppressive effect of SodSal (Table 6) Similar protecting effects were achieved by addition of serum albumin (of mouse and human origin) to RPMI at a final concentration of 2–4% Higher SodSal concentrations still showed significant inhibition of ^{35}S incorporation under full serum conditions although to a lesser extent than without serum (Table 1 vs Table 6) Since human serum has a relatively low sulfate concentration (0 3 mM) compared to mouse serum (1 0 mM), the ^{35}S incorporation values are higher in human serum owing to a higher specific activity ($^{35}S/[SO_4^=]$ (Table 6)

To study the influence of serum sulfate in detail, the effect of 2×10^{-3}M SodSal on patellar chondrocyte sGAG synthesis was tested at various sulfate concentrations in 100% mouse serum To this end mouse serum was depleted of sulfate with BaCl, and made up to the appropriate sulfate concentrations by addition of Na_2SO_4 As shown in Table 7, the therapeutic concentration of salicylate does not cause suppression of ^{35}S incorporation under serum conditions, either at a normal (1 0 mM) or at a severely depleted sulfate content (0 05 mM) On the other hand, the amount of sulfate *incorporation* was highly dependent on the sulfate *concentration* (Table 7, 2nd column) At 0 4 mM $SO_4^=$, the sulfate incorporation was already reduced to 60% of the value obtained at 1 0 mM $SO_4^=$

Ex vivo studies Groups of 5 mice received a single intraperitoneal injection of SodSal (200 mg/kg) or saline (controls) In drug treated mice the serum salicylate concentration was $2\,5 \times 10^{-3}$M at 30 min and slowly declined to $1\,2 \times 10^{-3}$M at 3 h, after 24 h no salicylate was detectable Endogenous inorganic sulfate in serum was reduced from 1 1 mM to 0 4 mM at 2 h and remained low at 3 h, at 24 h a normal (control) level (1 0 mM) was measured At various times (½, 1, 2, 3, 24 h) patellae were isolated and exposed immediately to ^{35}S-sulfate for 1 h Using such an *ex vivo* protocol, ^{35}S-sulfate incorporation values in patellae from SodSal treated mice were not found to be reduced compared to saline treated control mice at whatever time studied

Table 5 *SodSal (5×10 ⁻³M) effect on ³⁵S incorporation at high and low sulfate concentration after preincubation in high sulfate medium*

Drug	Premedium[§]	$^{35}SO_4$ medium	^{35}S incorporation[¶]	
	$[SO_4^=]$	$[SO_4]$	(CPM/Patella)	(% of Control)
Control	1 0 mM	1 0 mM	599 ± 126	
SodSal	1 0 mM	1 0 mM	424 ± 70	70
Control	1 0 mM	0 00003 mM	10394 ± 1629	
SodSal	1 0 mM	0 00003 mM	4548 ± 691	44[†]

§ Patellae were preincubated for 2 h in premedium (1 0 mM SO_4 before ^{35}S labeling was started (for another 2 h) in $^{35}SO_4$ medium with either 1 0 mM SO_4 or 0 00003 mM SO_4 During this whole incubation period (4 h) SodSal was present or not (control)
¶ Values represent mean ± SD of patellae from groups of 6 mice
† Significantly different from value obtained at high sulfate $p<0.01$ (unpaired 2 tailed)

Table 6 *Influence of full serum on the SodSal mediated inhibition of ^{35}S incorporation in whole patellae*

SodSal	CPM Patella (% of Control)§			
(Mol/l)	100% Mouse Serum	p†	100% Human Serum	p
Control	1066 ± 97 (100)		3580 ± 588 (100)	
1×10^{-3}	1094 ± 171 (103)	NS	3658 ± 743 (102)	NS
2×10^{-3}	1055 ± 210 (99)	NS	3477 ± 521 (97)	NS
5×10^{-3}	856 ± 122 (80)	<0.01	3086 ± 495 (86)	NS
1×10^{-2}	640 ± 26 (60)	<0.001	2359 ± 280 (66)	<0.001

§ Data represent mean ± SD of patellae from groups of 6 mice
† Statistical significance of difference from control (unpaired 2 tailed) NS = not significant

Table 7 *SodSal (2×10^{-3}M) effect on ^{35}S incorporation at variable sulfate concentrations in 100% normal mouse serum*

SO_4 conc	SO_4 incorp (control)	CPM Patella¶	
mM	mmol × 10^8	Control	SodSal
0.05	0.15 (10%)	1161 ± 243	1305 ± 287 (112%)§
0.2	0.5 (33%)	893 ± 196	993 ± 79 (111%)§
0.4	0.9 (60%)	814 ± 64	746 ± 90 (92 %)§
1.0	1.5 (100%)	546 ± 44	544 ± 130 (99 %)§

¶ Values represent mean ± SD of patellae from groups of 5 mice
† % of mmol sulfate incorporated at $SO_4 = 1.0$ mM
§ % of control not significantly different from control (unpaired 2 tailed)

DISCUSSION

In our patella assay, salicylate suppresses articular cartilage GAG synthesis, as measured by ^{35}S-sulfate and ^{3}H-glucosamine incorporation, in a dose dependent fashion However, therapeutic concentrations ($1\text{-}2 \times 10^{-3}$M) appear to have no significant effect if the assay is performed in the presence of 100% serum In a recent study we demonstrated that *in vivo* salicylate causes not only suppressed sGAG synthesis, but also a striking fall in the endogenous sulfate level[8] Although, the suppressive action of therapeutic salicylate concentrations tends to be stronger with decreasing sulfate levels in the incubation medium, sulfate deprived serum still fully protects cartilage against antimetabolic drug effects Consequently, if synovial fluid *in vivo* has the same protective effect as serum *in vitro*, the salicylate induced suppression of sGAG synthesis observed *in vivo*[8] is not due to a direct effect on the chondrocyte

Salicylate acts very fast Without serum, suppression of patellar sGAG synthesis was already demonstrated after one h's exposure to the drug (2×10^{-3}M) Whitehouse[14] showed in a study employing cartilage slices, that only 20 min were needed to establish this effect We found no aggravation of suppression when the exposure time was extended to 6 h Moreover, even at the high salicylate concentration of 10^{-2}M, the inhibition appeared completely reversible, since the sGAG synthetic rate returned to normal values within 2 h after removal of the drug

In view of the observations of Palmoski and Brandt[4], proteoglycan decreased cartilage slices may be more susceptible to the action of salicylate However, no evidence has been obtained that patellar cartilage with a decreased proteoglycan content is more vulnerable to salicylate Trypsin treated patellae or even patellae obtained from arthritic mice[9,10] do not show a higher inhibition upon exposure to salicylate compared with normal patella specimens (data not shown) We also investigated patellae from mice of different age (8, 15, 27 weeks) Although it is not known whether the patellar proteoglycan content is decreased with age as was found for human articular cartilage[15], drug mediated suppression of sGAG synthesis remained the same in every age group studied (Table 3) The observed decrease in ^{35}S incorporation with age is in agreement with previous studies[9]

An interesting finding was the repeatedly observed, albeit statistically insignificant, stimulation of sGAG synthesis at low salicylate concentrations (10^{-5} and 10^{-4}M) There are 2 publications on cartilage studies in which the same phenomenon is reported[2,16] Assuming that only free salicylate affects articular cartilage, it is interesting that at therapeutic levels ($1\text{-}2 \times 10^{-3}$M) the free, not protein-bound, salicylate concentration in biological fluids of patients is in this low range[17]

At a high salicylate concentration of 10^{-2}M, which causes severe suppression of ^{35}S-sulfate incorporation, no adverse effect was observed on the normal ^{35}S-GAG release in prelabeled patellar cartilage Studies of others, done on cartilage slices, revealed that salicylate either inhibited GAG breakdown (rabbit cartilage[18]) or even accelerated it (canine cartilage[2]) to varying degrees, depending on drug concentration In trying to explain these discrepancies there are several points to consider Comparing the above assay methods, differences exist in duration of tissue culture, prelabeling schedule, animal species, medium composition and cartilage integrity (anatomically intact versus damaged cartilage) Our study reports only on short term (hours) salicylate effects Recent data on this subject suggest that the outcome may be dependent on the culture conditions We have indications that just by prolonging the ^{35}S prelabeling period from 2 to 17 h, salicylate (10^{-2}M) causes some in-

hibition of the subsequent ^{35}S GAG release compared to control values from patellae undergoing the same incubation schedule without drug

We recently reported that SodSal, administered to mice in a dose of 200 mg/kg not only suppressed patellar sGAG synthesis *in vivo* but also induced a striking fall in serum sulfate from the normal level of 1 0 mM to less than 0 4 mM within 4 h[8] In subsequent *in vitro* experiments, we demonstrated that lowering of sulfate *per se* already provoked a decreased sGAG synthesis[8] To establish which mechanism underlies the observed *in vivo* suppression of sGAG synthesis (direct salicylate action, deprived sulfate availability or both) the influence of sulfate and serum on the salicylate mediated suppression was examined *in vitro* In the RPMI culture medium used in our studies, the sulfate concentration was 0 4 mM and this is rather low compared with the physiological serum sulfate level of 1 0 mM in C57B1 mice Employing these 2 sulfate concentrations, we observed that salicylate provoked a higher degree of suppression at low sulfate (Table 4) An important message from this observation is that one should make allowance for artificial drug effects in performing cartilage assays at too low sulfate levels Along this line it is common practice to assess sGAG synthesis by incorporation of ^{35}S sulfate in a sulfate poor culture medium in order to reach high incorporation values[19 20] In assaying salicylate, this schedule leads to an even higher suppression, which is actually an overestimated effect (Table 5)

Our findings indicate that at least in cartilage culture studies, one should preferably use sulfate concentrations also present in body fluids of species from which the cartilage tissues are derived The various commercial culture media as well as sera from several species differ markedly in sulfate levels[21] The lowest sulfate concentration is found in human serum (0 3 mM[22]) and the highest concentrations are found (among others) in sera of cows (1 8 mM[21]), rabbits (2 0 mM[21]), fetal calves (0 9 mM[22]), dogs (1 2–1 8 mM[21 24]) and mice of the C57B1 strain used in our study (1 0 mM) Presumably most cartilage studies to date were performed at nonoptimum sulfate concentrations At this point it is important to mention the many studies done by Palmoski and Brandt Starting about 7 years ago, they extensively investigated drug effects, particularly of salicylate, on canine articular cartilage As stated in one of their publications[25] the sulfate concentration in the culture medium (Ham's F12) was 5×10^{-6}M Despite the presence of 10% fetal calf serum (FCS) in the medium, the sulfate concentration is far too low compared to the level found in dogs (1 2–1 8 mM) Moreover, they incubated cartilage slices for more than 24 h, probably in this sulfate deprived medium[4] A possible overestimation of drug effects therefore cannot be ruled out

In the initial studies done to develop our patella assay[9], it was noticed that addition of 20% mouse serum to the culture medium did not stimulate or inhibit ^{35}S-sulfate incorporation, at least in short term incubations However, in studying NSAID in the therapeutic concentration range, it is very important to realize that *in vivo* most of the drugs are for more than 95% bound to plasma proteins (chiefly albumin) For salicylate the degree of binding (99–75%) appeared to be inversely proportional to the concentration ($0\,5\,2\,5\times10^{-3}$M) in human plasma[17] If the amount of free drug determines its activity to articular cartilage, then the effectiveness of salicylate is reduced by the extent of protein binding Since the free salicylate concentration in SF is virtually identical to that in plasma[26] it is plausible that equal concentrations of free drug will be obtained *in vitro* using 100% mouse serum as incubation medium for our patella assay Employing serum, the antimetabolic action of salicylate appeared to be remarkably reduced Therapeutic concentrations of salicylate, present in mouse serum as well as in human serum, seem not to inhibit the chondrocyte sGAG synthesis anymore (Table 6)

Many investigators working with cartilage cultures apply no more than 10 to 20% serum in the culture medium In performing drug studies these serum levels may be too low, since a higher fraction of the drug may remain in the free form compared to *in vivo* conditions, and consequently is expected to be more potent in its chondrocyte directed action Nevertheless studies done by us with mouse serum[8] and by others with FCS[5 27] demonstrated that 10–25% serum is apparently enough to completely abolish the suppressive effect of a low but therapeutic salicylate concentration (10^{-3}M) In one study this protective effect of serum (10% FCS) was observed only when anatomically intact knee cartilage from loaded portions of the joint was used[5 28], with intact cartilage from unloaded sites or with slices of normal or OA cartilage, 10^{-3}M salicylate continued to exert a moderate to marked suppressive effect on sGAG synthesis[25] At all events, our studies with albumin of human and mouse serum suggest that, perhaps among other unknown factors, the protective influence of serum may be attributable to protein binding of salicylate

As discussed before, it is evident that the suppressive effect of salicylate ($1–2\times10^{-3}$M) on patellar chondrocyte sGAG synthesis is more pronounced at relatively low sulfate concentrations, but also that this suppression is abolished by 100% serum In this respect it is important to note that chondrocyte sGAG synthesis is suppressed already by a decreased availability of environmental sulfate in mouse serum, which phenomenon was also demonstrated in our previous studies using sulfate deprived culture medium without serum[8] Reduction of sulfate incorporation in patellar cartilage is already manifest at 0 4 mM SO_4^{2-} (40% reduction) and markedly high at 0 05 mM SO_4^{2-} (90% reduction) Interestingly, despite this disturbed sulfate metabolism, salicylate (2×10^{-3}M) appeared to have no additive suppressive effect (Table 7) Thus, even at low sulfate concentrations,

the protective property of serum is completely preserved It is reasonable to assume that this protection is also true for SF which is in drug equilibrium with the blood circulation Consequently, the recently observed *in vivo* suppression of patellar sGAG synthesis by systemically administered salicylate[8] cannot be due to a direct drug effect on chondrocytes, but might be at least to some extent, attributable to a (salicylate induced) decrease in sulfate availability (< 0 4 mM SO_4)

The suppressed sGAG synthesis experienced by patellar cartilage *in vivo* upon exposure to low sulfate, is obviously a rapid and reversible phenomenon, since subsequent *in vitro* ^{35}S sulfate incorporation in patellae from salicylate treated mice was not decreased in comparison with patellae from saline treated mice The same kind of *ex vivo* studies were performed by Palmoski and Brandt[29] on articular cartilage of dogs treated for 9 weeks with therapeutic doses of aspirin They observed no adverse effect on sGAG synthesis by normal cartilage, which is consistent with results from our short term *ex vivo* studies However, OA cartilage showed severe suppression of sGAG synthesis *in vitro* upon this chronic *in vivo* drug treatment It is tempting to speculate that this inhibition of chondrocyte function is due to a salicylate induced chronically decreased sulfate availability, causing irreversible effects on OA cartilage

All our experiments were performed with normal anatomically intact, articular cartilage of the mouse patella and sodium salicylate as representative of the many NSAID available It would be of interest to assess whether the concentration of sulfate and the presence of serum also modulate effects of other drugs, and how this may be further influenced in diseased cartilage

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CHAPTER 6

IN VIVO AND IN VITRO EFFECTS OF TIAPROFENIC ACID ON GLYCOSAMINOGLYCAN SYNTHESIS BY INTACT MURINE ARTICULAR CARTILAGE

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In vivo and in vitro effects of tiaprofenic acid on glycosaminoglycan synthesis by intact murine articular cartilage

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Summary

A number of non-steroidal anti-inflammatory drugs have been shown to suppress chondrocyte glycosaminoglycan synthesis. In this study, the effects of tiaprofenic acid and sodium salicylate on $^{35}SO_4$ incorporation into intact patellar cartilage of C57 B1 mice, in vitro and in vivo, were investigated. The water-soluble trometamol salt of tiaprofenic acid was used for both incubation and intraperitoneal injection. After incubation with the equivalent of 5 or 20 mg/l of tiaprofenic acid, ^{35}S incorporation into patellar cartilage was slightly but not significantly increased. Trometamol alone had no effect. Sodium salicylate decreased incorporation of ^{35}S at a concentration of 1 mM (to 80% of control value) and to a greater extent at 5 mM (to 54% of control value). In the in vivo studies, single doses of 4, 8, 16 and 32 mg/kg of tiaprofenic acid were used. Trometamol on its own was tested at a dose of 15 mg/kg. Sodium salicylate was given at a dose of 200 mg/kg. Blood and patellar samples were taken two hours later and assayed for ^{35}S. Tiaprofenic acid given orally or i.p. had no effect on blood ^{35}S content but caused a slight reduction in ^{35}S incorporation. Sodium salicylate caused a marked decline in blood ^{35}S content, and this was probably responsible for the reduction in ^{35}S incorporation observed. Surprisingly, oral administration of the doses of tiaprofenic acid and sodium sali-

cylate mentioned above for seven and a half weeks had no deleterious effect on the knee-joint cartilage as was shown by histological examination.

Introduction

Several non-steroidal anti-inflammatory drugs (NSAIDs) commonly used in the treatment of rheumatic diseases provoke suppression of glycosaminoglycan (GAG) synthesis when slices of articular cartilage are cultured in the presence of these drugs [1-3].

We recently reported that salicylate, the most common NSAID, reduced GAG synthesis in normal articular cartilage of the mouse patella in vitro as well as in vivo. It is of interest in the latter case that a salicylate-induced depletion of endogenous sulphate was probably responsible for the observed suppression of sulphated GAG synthesis [4,5].

We describe here our findings from preliminary experiments on the in vitro and in vivo effects on normal articular cartilage of tiaprofenic acid (TA), a new NSAID. These studies were done using anatomically intact murine patellar cartilage [6].

Materials and methods

Animals

Normal male C57 Bl mice, 6 to 9 weeks of age and weighing 20 to 25 g, were used. They were matched for weight and age before the start of each experiment.

In vitro studies

Mice were sacrificed by cervical dislocation, and patella specimens were carefully removed from the knee-joints. The patellae were incubated as described [6] using RPMI culture medium containing the water-soluble trometamol salt of tiaprofenic acid (TroTA), trometamol (Tro) alone, sodium salicylate (SodSal) or no drug (control). After 30 min the patellae were transferred to fresh medium of the original composition plus $Na_2{}^{35}SO_4$ (10 μCi/ml). The incubation was continued for an additional 2 h. Thereafter, patellae were analysed for newly-formed cartilage GAGs. This was done by counting the amount of incorporated ^{35}S-sulphate [6], since it was shown that the ^{35}S content of whole patellae is a reliable measure for newly-formed sulphated GAGs in the patellar cartilage [7].

In vivo studies

Groups of mice were injected intraperitoneally (i.p.) with TroTa, Tro and SodSal (10 μl/g). Fifteen minutes later drug-treated mice and saline-treated controls received an i.p. injection of 2 μCi $Na_2{}^{35}SO_4$/g. At various time intervals blood samples (10 μl) were taken from the tail and assayed for radioactive content. Two hours after ^{35}S-sulfate administration mice were sacrificed and whole patellae isolated and processed for ^{35}S counting (see above).

Results and discussion

In vitro effect of tiaprofenic acid and sodium salicylate on ^{35}S-sulphate incorporation in patellar cartilage

The effect of TA was studied using the water-soluble TroTA in therapeutic concentrations. Whole patellae were incubated in drug-containing medium for 2.5 h, the final 2 hours in the presence of radio-labelled sulphate. Control patellae were cultured in the absence of drug.

As measured by ^{35}S incorporation, TroTA and Tro alone seemed to have no effect on the sulphated GAG synthesis in patellar cartilage (Table I). On the

Table I: In vitro effect of TA and SodSal on ^{35}S incorporation in patellar cartilage.

Group	Concentration	cpm/patella*	Percentage of control
Control	–	596 ± 130	100
TroTA	30 mg/l	680 ± 75	114
TroTA	7.5 mg/l	670 ± 163	112
TroTA	0.15 mg/l	541 ± 88	91
Tro	9.5 mg/l	617 ± 142	104
SodSal	5 mM	322 ± 68	54
SodSal	2 mM	465 ± 88	78
SodSal	1 mM	507 ± 127	85

TA = tiaprofenic acid
Tro = trometamol
TroTA = trometamol salt of tiaprofenic acid
SodSal = sodium salicylate
*Values represent mean ± SD of patellae from groups of at least 5 mice.

Table II: In vivo ^{35}S incorporation in patellar cartilage after TA and SodSal treatment.

Group	Concentration (mg/kg)	cpm/patella*	Percentage of control
Control	–	327 ± 38	100
Tro	15	334 ± 95	102
TroTA	48	244 ± 62	75
TroTA	24	250 ± 55	76
TroTA	12	230 ± 46	70
TroTA	6	239 ± 30	73
SodSal	200	197 ± 28	60

See Table I for abbreviations.
*Values represent mean ± SD of groups of 5 mice.

other hand, SodSal clearly exerted a dose-dependent suppression of ^{35}S-sulphate incorporation.

To examine the effect on the natural sulphated GAG breakdown in patellar cartilage, ^{35}S pre-labelled patellae were incubated with the highest concentrations of TA and SodSal (as given in Table I). No effect on the rate of GAG degradation in the cartilage was observed.

It should be emphasized that in these experiments no serum or proteins were added to the incubation medium, so the drugs were in the free form. Binding to serum proteins would reduce the concentration of unbound drugs. We found that 25% or more normal mouse serum in the culture medium offered complete protection for the suppressive action of 1 and 2 mM SodSal, concentrations which are achieved in the serum of patients [7].

With the results of these in vitro experiments it would be anticipated that these drugs would have no effects on GAG metabolism in normal articular cartilage in vivo at least when applied in a short-term schedule.

Effect of tiaprofenic acid and sodium salicylate on in vivo ^{35}S-sulphate incorporation in patellar cartilage

Groups of mice were treated with a single dose of Tro, TroTa or SodSal, and control groups received saline. Fifteen minutes after administration of the drug (or saline) mice were injected with radio-sulphate. Two hours later mice were sacrificed and patellae isolated.

TroTa or Tro alone had a negligible effect on the ^{35}S content of the blood

compared with control values. In contrast SodSal caused a clear decline in the blood ^{35}S content (up to 40% of control values). The amount of ^{35}S-sulphate incorporated in the patellae of drug-treated mice was in all cases reduced as compared with saline- or Tro-treated controls (Table II). About the same results were obtained after application of TA in pure form per os.

The effects were not irreversible. The suppressed uptake of ^{35}S-sulphate seen immediately after TroTa or SodSal administration had already returned to normal 5 hours after drug application.

As found in earlier studies [4,5], the decrease in ^{35}S content in the blood caused by a SodSal-induced sulphate diuresis appeared to be responsible for the reduced incorporation of ^{35}S in the patellar cartilage GAGs. It is difficult to interpret the data obtained for TA. There is no dose-response effect observable with TA; all doses seem to suppress the ^{35}S-incorporation in patellar cartilage to the same extent. Moreover, since TA appeared not to influence the ^{35}S handling in the mouse, a decreased sulphate availability cannot be the reason for the reduced ^{35}S uptake in cartilage. A direct effect of TA on the patellar chondrocyte seems unlikely since short-term in vitro studies showed no effect on sulphated GAG metabolism (Table I). At the moment, there is no reasonable explanation for this in vivo observation, and it is currently under investigation.

Above all, it is very important to realize that in vivo drug effects can be totally different from those found in vitro.

Effect of chronic oral administration of tiaprofenic acid and sodium salicylate on mouse knee-joint cartilage

Groups of mice received once-daily oral doses for $7\frac{1}{2}$ weeks of TA (32, 16, 8 and 4 mg/kg), SodSal (200 mg/kg) or water (controls). Twenty-four hours after the last drug application, mice were injected with radio-sulphate. Three hours later the mice were sacrificed and the knee joints removed in toto for histological and autoradiographic studies.

Sections of whole knee joints, from TA- as well as from SodSal-treated mice, showed no chondrocyte death or depletion of cartilage proteoglycans as measured with Safranin O staining. As indicated by the labelling intensity due to incorporated ^{35}S (autoradiographs), the vitality of the articular chondrocytes was not affected, as compared with chondrocytes in control sections.

Conclusions

In view of the data obtained in short-term in vivo experiments (Table II), it

is surprising that on gross observation the mouse knee-joint cartilage seems normal in every aspect The effects of chronically administered drugs on articular cartilage will now be studied in more detail

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CHAPTER 7

EFFECTS OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS ON THE METABOLISM OF SULFATED GLYCOSAMINOGLYCANS IN HEALTHY AND (POST)ARTHRITIC MURINE ARTICULAR CARTILAGE IN VITRO AND IN VIVO

Bernard J de Vries, Wim B van den Berg, Elly Vitters and Levinus BA van de Putte

EFFECTS OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS ON THE METABOLISM OF SULFATED GLYCOSAMINOGLYCANS IN HEALTHY AND (POST)ARTHRITIC MURINE ARTICULAR CARTILAGE IN VITRO AND IN VIVO

ABSTRACT

A number of nonsteroidal antiinflammatory drugs (NSAIDs) were studied for their effects on normal and damaged murine articular cartilage, both in vitro and in vivo. In vitro, in the absence of serum, sodium salicylate (SodSal) caused significant suppression of ^{35}S-glycosaminoglycan (GAG) synthesis, whereas tiaprofenic acid (TA), piroxicam (PX), prednisolone sodium phosphate (Pred) and several other NSAIDs were without effect. Trypsin-mediated proteoglycan depletion did not change the susceptibility of the articular chondrocyte to these drugs. When arthritic cartilage was taken from an acutely inflamed joint also no aggravation of drug effect was seen and Pred even seemed to diminish inflammation mediated suppression of ^{35}S-GAG synthesis.

Short-term in vivo effects of some drugs were tested in mice with unilateral zymosan induced arthritis. At day 1 after arthritis induction, in vivo ^{35}S-GAG synthesis by the cartilage of the arthritic joint was decreased to 63%. Only SodSal suppressed in vivo ^{35}S-GAG synthesis in the healthy and arthritic joint, both to the same extent. At day 28, GAG synthesis in the postarthritic joint was enhanced to 160%. This type of cartilage appeared to be more susceptible to drug effects, since all drugs tested showed clear suppression of the augmented GAG production in vivo. Finally, in vivo drug effects were tested on normal and enhanced ^{35}S-GAG degradation, the latter in the zymosan induced arthritic joint. Both TA and Pred appeared to suppress degradation in healthy and, to some extent, in arthritic cartilage. In conclusion, of the drugs tested only salicylate seems potentially harmful towards articular cartilage, piroxicam showed no adverse effect and tiaprofenic acid and prednisolone even exerted some beneficial effects.

INTRODUCTION

A serious drawback of the use of NSAIDs in the treatment of arthritis or arthrosis (osteoarthritis) may be their potential side effect on articular cartilage. Using in vitro culture systems with slices of normal articular cartilage, suppression of GAG synthesis was demonstrated first for salicylate (1) and subsequently for a number of other NSAIDs (2). Later on, it was shown that the suppressive effect of salicylate was aggravated when arthrotic cartilage was used compared to normal cartilage (3). In addition, long-term in vivo treatment with salicylate enhanced the extent of cartilage damage in experimental arthrosis models (4,5) and also the occurrence of spontaneous arthrosis in a susceptible mouse strain (5,6).

Using anatomically intact murine articular cartilage we found no harmful effect of salicylate on basal GAG metabolism under nearly physiological in vitro conditions (7). Furthermore, we provided evidence that in vivo suppressive effects, provoked by salicylate administration to healthy mice, could be related to a disturbance in the endogenous sulfate pool rather than to a direct influence on articular chondrocytes (8). In the present study we compared a number of NSAIDs for their effects on normal and damaged cartilage both in vitro and in vivo.

MATERIALS AND METHODS

Animals. Male C57Bl10 mice, aged 9-12 weeks at the start of each drug experiment were used. They were fed a standard, pellet diet and tap water ad libitum. They were in good health or suffered from monoarthritis, which was induced by intra-articular injection of 180 µg zymosan in the righ knee joint. Prior to the start of each experiment, mice (22-27 gram) were weight-matched (± 2 g) and age-matched (± 0.5 week).

Antirheumatic drugs. Tiaprofenic acid (TA) was obtained from Roussel Uclaf, Piroxicam (PX) from Pfizer, Indomethacin and Sulindac sulfoxide from Merck, Sharp and Dohme, Naproxen-sodium salt from Sarva-Syntex, Ibuprofen from the Boots Company, Pirprofen, Tribenoside and CG 53918 (an experimental drug) from Ciba Geigy. Sodium salicylate (SodSal),

Acetyl salicylic acid, Paracetamol and Prednisolone sodium phosphate (Pred) were delivered by our in-house pharmacy.

In vitro studies. Patellar cartilage was used as a source of anatomically intact articular cartilage and sulfated GAG metabolism was studied by described methods (7,10,11). In the presence of drug in the culture medium (RPMI), most incubations were performed in a time-span of 4 hours of which the last 2 hours with ^{35}S-sulfate (20 µCi/ml). The amount of radiosulfate was expressed in radioactivity units. Incorporation values were adjusted to the mean control value (incubation without drug), which was accepted as 100 units. The drugs were directly dissolved in RPMI culture medium, or first in 0.15 N NaOH (TA, PX) or ethanol (ibuprofen). During incubation the pH was 7.4 and ethanol, if present, below 0.1%.

In some experiments normal patellae were first subjected to trypsin (1 mg/ml RPMI) treatment for one hour (37 °C), in order to deplete the cartilage matrix of proteoglycans. After thorough washing with RPMI they were used for the described drug incubation experiments.

In vivo studies. Groups of mice with 1 day-lasting monoarthritis received drugs orally in solution or in suspension in tap water by gastric intubation. Control mice received tap water (250 µl/mouse). To study drug effects on in vivo sulfated GAG synthesis, $NA_2{}^{35}SO_4$ was injected intraperitoneally (2 µCi/g) 30 minutes after drug dosage. Four hours later mice were killed and the incorporated amount of ^{35}S-GAG in their patellae was assessed (10,11). For in vivo degradation studies, groups of mice were first injected with ^{35}S-sulfate followed, 24 hours later, by drug administration. Two hours after drug application unilateral zymosan arthritis was induced. At this time point the amount of incorporated ^{35}S in the patella was accepted as 100 units. Then, drugs were given 4 hours later and, at the next day, 22 hours later (i.e. day 1 arthritis). In one experiment, drug dosing was continued with a fourth and fifth dosage, 28 and 46 hours after induction of arthritis (i.e. day 2 arthritis), respectively. Mice were killed two hours after the last drug application. The remaining ^{35}S amount in the healthy and arhritic patellae was subsequently quantified.

Statistical analysis. Differences between means were considered statistically significant when $p<0.05$ according to Student's t test for unpaired data. Since the standard deviation was less than 20 or, in some experiments, 25 ^{35}S-radioactivity units, the control values (mean ±SD) were not always stated apart, but accepted as e.g. 100 ± 25 units.

RESULTS

In vitro effect of various drugs on normal anatomically intact cartilage. The effect of a number of drugs on chondrocyte ^{35}S-GAG synthesis was studied in a short-term in vitro culture. Murine patellae were preincubated in drug-containing medium for 2 h followed by a 2 h ^{35}S-sulfate labeling period in the presence of the drug. As described earlier (7) SodSal caused a dose dependent suppression of ^{35}S-GAG synthesis in the concentration of 1-5 mM. The highest dose was routinely used as a positive control for drug screening in our cartilage assay system. At therapeutic antiinflammatory concentrations and higher ones, neither TA, PX nor Pred caused a significant change in ^{35}S-GAG synthesis (Table 1). A number of other drugs listed in Table 1 were tested once. Only aspirin and at a very high concentration, pirprofen seem to inhibit chondrocyte synthetic function. Ethanol, a vehicle commonly used to dissolve certain drugs, can safely be used in end concentrations of 0.04-0.1%, but higher concentrations suppressed chondrocyte GAG synthesis. The lowest concentrations of TA, PX and Pirprofen (Table 1) occur as free (i.e. not plasma-bound) amounts in the synovial fluid of therapeutically treated patients. Around these low concentrations we never observed adverse effects on patellar GAG synthesis. SodSal, PX, TA and Pirprofen were also tested at several concentrations, including those of Table 1, in ^{35}S-pulse chase experiments (2 h pulse and 6 h chase) to examine their potential effect on the basal PG breakdown. Neither retardation nor acceleration of PG catabolism was observed. In further in vitro experiments, to be described here, the effects of SodSal, TA, PX and Pred were investigated only at the highest concentration shown in Table 1.

Examination of potential changes in drug susceptibility of damaged cartilage in vitro. Joint inflammation is accompanied by proteoglycan de-

Table 1. Effect of various NSAIDs on basal sulfated GAG synthesis in mouse patellar cartilage in vitro.

Drug	Concentration		^{35}S incorporation	N °
	mM	µg/ml	units#	
Sodium salicylate (SodSal)	5.0	800	62 ± 13*	10
Tiaprofenic acid	3.8×10^{-4}	0.1	98 ± 16 /121 ± 24	2
(TA)	7.7×10^{-2}	20	99 ± 20	6
Piroxicam	6.0×10^{-4}	0.2	102 ± 23 / 90 ± 14	2
(PX)	1.2×10^{-1}	40	97 ± 12	6
Prednisolone sodium phosphate (Pred)	3.0×10^{-1}	145	98 ± 7	4
Acetylsalicylic acid (Aspirin)	4.4	800	74 ± 18*	1
Paracetamol	5.3	800	103 ± 14	1
Indomethacin	1.4×10^{-3}	0.5	95 ± 28 /119 ± 15	2
Naproxen, sodium salt	1.6×10^{-1}	40	105 ± 28	1
Sulindac sulfoxide	5.6×10^{-3}	2	118 ± 29	1
Ibuprofen	1.9×10^{-1}	40	95 ± 12	1
Pirprofen	4.0×10^{-3}	1	102 ± 18	1
	1.5×10^{-1}	40	72 ± 8*	1
Ethanol	0.04 %	40	101 ± 18	1
	0.1 %	100	104 ± 21	1
	0.4 %	400	73 ± 17*	1

Results represent the mean ± SD of incorporated radioactivity units/patella (see materials and methods), calculated from data of one or more experiments, each consisted of patellae from 6 mice. If more than two experiments were done the mean of the average incorporation values per experiment is presented. Mean control value is accepted as 100 ± 20 units.

* Significantly different from control.

° Number of experiments.

pletion of the cartilage matrix, and a suppressed chondrocyte synthetic activitity (9). To investigate whether chondrocytes in a proteoglycan (PG) depleted matrix per se are more susceptible to adverse drug effects, murine patellae were artificially depleted of PGs using short-term trypsin treatment. This resulted in loss of 40-50% of the PGs and the subsequent chondrocyte synthetic activity was reduced to 32-43% of synthesis in normal cartilage. Nevertheless we did not observe any increase in suppressive effects of the drugs tested. Compared to normal cartilage, SodSal caused a similar or even a reduced suppression of sulfated GAG synthesis in trypsin treated patellae. Of interest, Pred

Table 2. In vitro effect of drugs on sulfated GAG synthesis by PG depleted cartilage.

Drug	N°	^{35}S incorporation units#	
		Normal	Trypsin
Control	1	100 ± 17	100 ± 10
	2	100 ± 17	100 ± 12
	3	100 ± 19	100 ± 18
Sodsal	1	46 ± 9*	63 ± 11*
	2	51 ± 8*	56 ± 8*
TA	1	83 ± 18	102 ± 27
	3	108 ± 27	90 ± 20
PX	2	79 ± 18	87 ± 21
Pred	1	96 ± 16	114 ± 8*
Relative ^{35}S content in absence of drug:		100%	38%

Radioactivity units per patella (see materials and methods). Values represent the mean ± SD of patellae from 6 mice. Mean control value accepted as 100 units.
* Significantly different from control.
° Experiment number.

appeared to stimulate slightly the suppressed rate of synthesis in the affected cartilage. TA and PX were still without effect (Table 2).

To mimic more closely the in vivo situation of an inflamed joint, patellae were taken from arthritic joints at day 1 and day 28 after induction of zymosan arthritis. At day 1 chondrocyte sulfated GAG synthesis was reduced to 51%. Again, on the average, no aggravation of the SodSal mediated suppression was found and, of the other drugs tested, Pred even significantly enhanced the synthetic activity in the arthritic cartilage (= relief of inhibition), (Table 3). At day 28, zymosan induced arthritis has waned (9) and the chondrocyte tries to restore its depleted matrix, reflected by enhanced synthesis (147%). Also this condition of the cartilage did not result in enhanced susceptibility to adverse drug effects (Table 3).

In vivo effects of drugs on healthy and arthritic cartilage. Unilateral arthritis was induced with zymosan to enable investigations of in vivo drug effects on sulfated GAG turnover in both normal and arhritic cartilage. In vivo, antirheumatic drugs may potentially exert adverse effects on already damaged cartilage but, on the other hand, may cause antiinflammatory effects, with concomitant reduction of inflammation mediated cartilage damage. Thirty minutes after oral administration of drugs, chondrocyte sulfated GAG synthesis was assessed in vivo by isolation of patellae 4 h after ^{35}S-sulfate injection. In healthy cartilage significant suppression of sulfated GAG synthesis was found after SodSal treatment, but not with the other drugs (Table 4). In arthritic cartilage, taken at day 1, ^{35}S-GAG synthesis is already reduced to 63% compared to synthesis in contralateral healthy cartilage. SodSal mediated suppression of sulfated GAG synthesis was also found in this arthritic cartilage and appeared to be in the same order of magnitude (Table 4). Again the other drugs (TA, PX, Paracetamol) showed no significant effect. At day 28 of zymosan arthritis the ^{35}S-GAG synthesis was enhanced to 160% in the postarthritic cartilage compared to healthy cartilage. Again, although not as high as usual, SodSal mediated suppression of synthesis was found in the healthy cartilage. However, in the postarthritic cartilage the SodSal suppressive effect seemed slightly higher,

Table 3. In vitro effects of drugs on sulfated GAG synthesis by healthy and (post)arthritic articular cartilage.

Drug	N °	^{35}S incorporation units#			
		Day 1		Day 28	
		healthy	arthritic	healthy	postarthritic
Control	1	100 ± 14	100 ± 7	100 ± 20	100 ± 25
	2	100 ± 15	100 ± 28	100 ± 19	100 ± 21
SodSal	1	56 ± 4 *	67 ± 7 *	61 ± 10*	53 ± 11*
	2	69 ± 12*	46 ± 21*	86 ± 12*	68 ± 10*
TA	1	83 ± 18	102 ± 20	77 ± 14*	92 ± 19
	2	121 ± 13*	127 ± 42	122 ± 25	125 ± 23
PX	1	99 ± 16	96 ± 12	86 ± 21	80 ± 28
	2	94 ± 19	101 ± 27	119 ± 27	99 ± 28
Pred	1	95 ± 11	118 ± 12*	92 ± 17	96 ± 12
	2	109 ± 19	148 ± 14*	102 ± 20	120 ± 18
Relative ^{35}S content in absence of drug:		100 %	51 %	100 %	147 %

Values represent the mean ± SD of patellae derived from 6 mice with unilateral zymosan induced arthritis. Mean control values accepted as 100 units (see materials and methods).
* Significantly different from control.
° Experiment number.

Table 4. In vivo effects of drugs on sulfated GAG synthesis by healthy and (post)arthritic articular cartilage.

Drug	Dose (mg/kg)	^{35}S incorporation units# Day 1 healthy	Day 1 arthritic	(n)	Day 28 healthy	Day 28 postarthritic	(n)
Control•	-	100 ± 25	100 ± 25	(10)	100 ± 25	100 ± 25	(6)
SodSal	200	62 ± 14*	58 ± 14*	(10)	72 ± 8*	54 ± 5*	(6)
TA	16	86 ± 22	82 ± 24	(10)	95 ± 24	73 ± 22	(6)
PX	10	88 ± 24	88 ± 24	(10)	110 ± 17	57 ± 7*	(6)
Paracetamol	200	119 ± 30	113 ± 21	(8)	nd	nd	
Relative ^{35}S content in absence of drug:		100 %	63 %		100 %	160 %	

Data represent the mean ± SD of patellae derived from (n) mice with unilateral zymosan induced arthritis; nd = not done.
• Separate control experiments were performed for each drug tested. The mean ± SD of control values were accepted as 100 ± 25 units (see materials and methods).
* Significantly different from control values.

and PX and TA also caused inhibition, albeit the latter not significant, indicating that this cartilage is more drug susceptible. In fact, the drugs inhibit the attempt at repair of the cartilage by reducing the enhanced synthetic function (aimed to restore the PG depleted matrix).

Effect on in vivo breakdown of articular GAGs. Using unilateral zymosan induced arthritis the effect of drugs was tested on both basal and accelerated degradation in the healthy and arthritic joint, respective-

ly. Table 5 shows that 26-39% and 47% of the ^{35}S label was lost from the healthy patellae after 24 h and 48 h respectively, reflecting a rapid turnover. In the arthritic joint these figures were 49%-64% and 67% respectively, pointing to enhanced degradation. From the drugs tested TA, Tribenoside and CG 5391B significantly suppressed the basal breakdown over 24 h, as found in mice treated with water alone. However, compared to the original ^{35}S content (24 h before) Pred and PX (once) stopped significant breakdown in healthy cartilage. TA, CG 5391B and Pred (once) also had a significant reducing effect on enhanced degradation in the arthritic joint (Table 5).

Surprisingly, none of the drugs modulated the natural or accelerated breakdown *in the 2-days drug experiment. In this particular experiment* the antiinflammatory effect of the drugs was determined prior to the sacrifice of the mice. Therefore, joint inflammation was quantitated at day 2 arthritis by performing ^{99m}Tc-uptake measurements (9). Only Pred showed a significant antiphlogistic effect (Table 6).

Table 6. Drug effect on joint inflammation: ^{99m}Tc-uptake measurements.

Drug treatment	Day 2 zymosan-induced arthritis R/L-ratio#
Control	1.57 ± 0.09
SodSal	1.83 ± 0.29
TA	1.64 ± 0.19
PX	1.52 ± 0.22
Pred	1.24 ± 0.22*

Results depict the mean ± SD of R/L-ratios, calculated from the ^{99m}Tc-uptake (see ref 9) measured alternately on the right (R) knee joint (arthritis) and the left (L) kee joint (normal) of 6 mice.
* Significantly different from control ($p<0.01$) by the one-tailed Mann-Whitney U test.

Table 5. In vivo degradation of sulfated GAGs: ^{35}S content left in pre-labeled patellae.

Drug (single dose/kg)	Remaining ^{35}S content (units)#					
	N°	Day 1		N°	Day 2	
		healthy	arthritic		healthy	arthritic
Tap water (10 ml)	1	67 ± 14	44 ± 15	1	53 ± 12	33 ± 9
	2	61 ± 15	36 ± 8			
	3	74 ± 14	51 ± 9			
SodSal (200 mg)	1	64 ± 3	45 ± 5	1	63 ± 9	33 ± 3
	3	76 ± 11	44 ± 8			
TA (16 mg)	1	80 ± 20	70 ± 24*	1	64 ± 18	32 ± 7
	2	101 ± 30*	54 ± 16*			
	3	101 ± 22*	67 ± 20			
PX (10 mg)	1	52 ± 14	32 ± 8	1	64 ± 7	31 ± 10
	3	95 ± 29	58 ± 16			
Pred (1 mg)	2	82 ± 26	58 ± 13*	1	62 ± 8	40 ± 8
	3	89 ± 40	58 ± 21			
Tribenoside (200 mg)	2	99 ± 35*	55 ± 24		nd	nd
CG 5391B (25 mg)	2	94 ± 15*	53 ± 16*		nd	nd

Results represent the mean ± SD of patellae obtained from 6 monoarthritic mice; ^{35}S content at time of arthritis induction accepted as 100 ± 20 units; nd = not done.
* Significantly different from tap water treated mice.
° Experiment number.

Our data indicate that, of the NSAIDs tested, only salicylate exerted harmful side effects towards articular cartilage, whereas tiaprofenic acid (TA), piroxicam (PX) and prednisolon (Pred) did not. Furthermore, arthritic cartilage with a depleted matrix and changed chondrocyte synthetic activity did not merely show a higher susceptibility to adverse drug effects.

Earlier work from our laboratory revealed that salicylate mediated suppression of in vivo ^{35}S-GAG synthesis was probably related to a lowering of endogenous sulfate. Sulfated GAG synthesis is highly dependent on the sulfate concentration (7,8) and salicylate caused a deep fall in the serum sulfate level early after administration (8). Some of the other drugs tested in vivo in the present study, showed a variable, but ignorable effect on endogenous sulfate (data not shown).

Recent studies of Palmoski and Brandt demonstrated that suppression of GAG synthesis by salicylate and indomethacin was higher in arthrotic compared to normal cartilage (12). Not the PG depletion of the cartilage per se, with concomitantly higher drug accessibility to the chondrocytes (12), appeared to be resposible for this phenomenon but the apparent condition of the chondrocyte. Increased susceptibility was found in arthrotic depleted cartilage with highly enhanced chondrocyte synthetic activity (13). Our data lend further support to this. Mere enzymatic PG depletion of the cartilage matrix did not result in higher drug effects, and higher susceptibility was also not found in arthritic cartilage with a depleted matrix and already suppressed ^{35}S-GAG synthesis. However, our data in postarthritic cartilage do suggest that the phenomenon of enhanced drug susceptibility only occurs when the chondrocyte synthetic function is stimulated. Of interest, since this phenomenon was seen in vivo, and not in vitro, modulation of systemic factors by the drugs in question must be involved in the suppression of the augmented chondrocyte anabolic activity. It remains a matter of debate whether suppression of enhanced synthesis to nearly normal synthetic levels (Table 4) must be termed a harmful side effect. A final judgement seems related to the fact whether enhanced synthesis should be considered as a necessary attempt at repair or may be an unwanted overshoot phenomenon.

The maintenance of an intact cartilage matrix depends on the delicate balance of chondrocyte mediated synthesis and degradation of matrix components. Of the drugs tested in this study only salicylate appeared to inhibit synthesis. ^{35}S-pulse chase studies revealed that the present NSAIDs did not affect in vitro proteoglycan degradation in normal cartilage. However, in vivo some of the drugs like TA, Tribenoside and CG 5391B, and perhaps Pred clearly inhibited normal breakdown. Whether this should be considered as an unwanted side effect is at present unclear. In addition, these drugs also seemed to inhibit the enhanced degradation in arthritic cartilage in vivo, and this is undoubtedly a beneficial effect. Enhanced degradation is probably the result of protease activity in the cartilage, originating either from inflamed synovium, stimulated leucocytes or interleukin-1 (Il-1) activated chondrocytes (14,15). In vitro studies already demonstrated that steroids may interfere with cartilage breakdown by inhibiting Il-1 production (16).

The usefulness of an antirheumatic drug in the treatment of arthritis must be seen in the light of its overall effect. On the one hand drugs may be harmful when they further suppress the already decreased chondrocyte synthetic activity in arthritic cartilage. On the other hand drugs may suppress inflammation, and thereby inflammation mediated effects on cartilage such as inhibited chondrocyte PG synthesis and enhanced matrix degradation. Although NSAIDs are developed and applied for their antiinflammatory action, their effects in suppression of murine joint inflammation were not evident. In our hands only prednisolone significantly inhibited edema formation as measured by ^{99m}Tc-uptake by the joint (Table 6). Our findings are in agreement with observations of Hunneyball in the antigen-induced arthritis model in mice. They observed a similar lack of effect of a number of NSAIDs, in either inflammation or cartilage damage (17). When clear antiinflammatory action is lacking, the effect of NSAID treatment is probably primarily analgesic and the drug of first choice should be one without side effects on cartilage.

The place of steroids in the treatment of arthritis is still a matter of considerable debate. In this study prednisolone sodium phosphate diminished joint inflammation (Table 6), partly prevented inflammation mediated suppression of GAG synthesis in vitro (Table 3) and inhibited

enhanced degradation in arthritic cartilage in vivo (Table 5). Beneficial steroid effects were also seen in the study of Hunneyball (17). Inhibitory effects on chondrocyte synthetic activity as described in the literature (18,19) were not evident in the present study. It should be mentioned that the described inhibitory effects relate to (rather) high steroid doses. In this respect it seems worthwhile to extend steroid studies with low doses and to screen in long-term studies for unwanted side effects. Especially the observation that very low concentrations of steroids already inhibit in vitro Il-1 mediated cartilage breakdown (20) warrants further investigation of in vivo applicability, since side effects are then hardly expected.

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CHAPTER 8

EFFECT OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS ON CARTILAGE DESTRUCTION IN ANTIGEN INDUCED ARTHRITIS IN MICE

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EFFECT OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS ON CARTILAGE DESTRUCTION IN ANTIGEN INDUCED ARTHRITIS IN MICE

ABSTRACT

The nonsteroidal antiinflammatory drugs, salicylate, piroxicam and tiaprofenic acid and the steroid prednisolone were investigated in a long-term study for their potential detrimental or benificial effects on joint cartilage of mice with antigen induced monoarthritis. Daily drug treatment over a period of 4-7.5 weeks did not affect the histological characteristics of normal joints at all. Articular chondrocyte synthetic activity was even stimulated after salicylate and tiaprofenic acid treatment, but the significance of this finding is not clear yet. Cartilage damage, caused by inflammation in the knee joint, was neither markedly deteriorated nor attennuated by these drugs. Slight antiinflammatory properties (decrease in edema (^{99m}Tc-uptake) and in inflammatory cells) were only evident with prednisolone, piroxicam and salicylate.

INTRODUCTION

Antigen-induced arthritis is an allergic type of joint inflammation, elicited by intra-articular injection of antigen into the knee joint of pre-immunized animals. This type of arthritis can be induced in mice with positively charged antigens like methylated bovine serum albumin (mBSA) (1,2,3). It bears histopathological resemblance to rheumatoid arthritis with regards to lymphocytic infiltrate in the synovial tissue and progressive cartilage destruction (4,5). One pharmacological study in this murine model so far has shown that the arthritis responds to steroids and some second line drugs (6,7). The nonsteroidal antiinflammatory drugs (NSAIDs), ibuprofen, flurbiprofen and indomethacin were without effect (6).

NSAIDs are commonly used in the treatment of various forms of arthritis, including rheumatoid arthritis. However, recent experimental studies have shown that certain NSAIDs are detrimental to articular cartilage, especially osteoarthritic cartilage (8,9). On the one hand, drugs may theoretically exert beneficial effect in arthritis by suppressing joint inflammation, and therefore inflammation mediated cartilage damage (4,5). On the other hand, chondrocytes in already damaged cartilage of an arthritic joint perhaps may be more susceptible to adverse drug effects.

The present study was set up to investigate the overall effect of long-term treatment with various NSAIDs in the murine arthritis model. Animals were used with unilateral arthritis, which enables us to screen the effect of drugs on both normal and arhritic cartilage.

MATERIALS AND METHODS

Animals and arthritis induction. Healthy, male C57Bl10 mice, aged 6-7 weeks, were used for a 7.5 week drug treatment experiment (salicylate and tiaprofenic acid). Unilateral arthritis was induced in mBSA-immunized mice (9-12 weeks of age) as previously decribed (5) by intraarticular (i.a.) injection of 40 µg mBSA (in 6 µl physiological saline) into the right knee joint. These inflammatory monoarthritic mice were used to study the effects of oral administered drugs, during a 4 week period, on both normal and arthritic cartilage. To assess the severity

of knee joint inflammation, ^{99m}Tc-uptake measurements were done as described (10). Mice were fed a standard, pellet diet and tap water or drug-containing water (see below) ad libitum. Prior to the start of each experiment, mice were weight- and age-matched.

Drugs. Mice received by oral gavage 250 µl of tap water (control) with dissolved drugs (prednisolone sodium phosphate, sodium salicylate) or with homogenically suspended drugs (piroxicam, tiaprofenic acid). In another experiment drugs were added to the drinking water. The drugs were dissolved in tap water or first in a negligible volume (less than 0.5% of the ultimate volume of drug water) of 0.15 N NaOH. All drug-waters had pH 8, identical to the pH of tap water at that time. Drink containers were protected from light; contents (70 -150 ml) were routinely renewed every 24 h.

Histology and autoradiography. Mice were killed by ether anaesthesia. The joints were dissected and processed for histology. Total knee sections (7 µm) were prepared, mounted on gelatin-coated slides and stained with haematoxylin and eosin (H & E) or with Safranin O. When chondrocyte function was studied, mice were injected intraperitoneally with 2 µCi $Na_2{}^{35}SO_4$/g 3 h before sacrifice. Paraffin sections of total knee were dipped in K5 emulsion (Ilford, Basildon, Essex, England) and exposed for 3 weeks. After this period the slides were developed and stained with H & E. Cellular infiltration, exudation and proteoglycan loss (Safranin O loss) were scored semi-quantitatively with light microscopy on five to seven serial sections.

In some experiments the patellae of ^{35}S-sulfate treated mice were excised and used to quantitate chondrocyte synthetic activity (11).

RESULTS AND DISCUSSION

Effect of NSAIDs on developing arthritis. Unilateral arthritis was induced in mBSA immunized mice by i.a. injection of 40 µg mBSA into the right knee joint. Drug dosing commenced four hours prior to arthritis induction. From that moment, drugs were given twice a day (at 9 h a.m.

and 6 h p.m.), seven days a week for four weeks by oral application. At days 7, 14 and 21 ^{99m}Tc-uptake measurements were done to assess the severity of knee joint inflammation. Inflammation was expressed as the ratio of ^{99m}Tc-uptake in the right arthritic versus that in the left non-inflamed joint. At day 28 animals were sacrificed and inflammation and cartilage destruction were scored on whole joint sections. Table 1 shows the effects on ^{99m}Tc-uptake. None of the drug treated groups showed a significant suppression compared to the group treated with water. However, group 1, i.e. mice not treated with daily gavage with water, showed significantly higher inflammation at day seven.

Histologic assessment of joint inflammation and cartilage damage is shown in Table 2. No large differences were seen between the various groups with respect to infiltrate, exudate and proteoglycan loss from the articular cartilage. The left non-arthritic joints of all groups were completely normal in every aspect, indicating that 4 weeks drug treatment does not anyhow cause gross cartilage damage.

The previous experiment suggested that the act of oral administration, twice a day, on its own may suppress joint inflammation. Group one showed higher Tc-uptake at day 7 and cartilage proteoglycan deple-

Table 1. ^{99m}Tc-uptake measurements.

			R/L ratio of ^{99m}Tc-uptake#		
Group	Treatment	Dose •	Day 7	Day 14	Day 21
1	-	-	1.47±0.18*	1.18±0.08	1.17±0.10
2	water	-	1.29±0.14	1.18±0.08	1.11±0.11
3	sodium salicylate	200	1.32±0.10	1.20±0.08	1.14±0.08
4	piroxicam	10	1.24±0.13	1.14±0.10	1.12±0.11
5	tiaprofenic acid	16	1.34±0.19	1.16±0.14	1.17±0.10

R/L ratio's exceeding 1.1 indicate joint inflammation; data represent mean±SD of 12 mice.
• Dose of one oral application in mg/kg.
* Significant difference between group 1 and 2: $p<0.02$.

Table 2. Histological grading of arthritis.

Group	Treatment	Infiltrate in* synovium	Exudate in* joint space	Cartilage PG • depletion
1	-	1.1#	0.8	1.8
2	water	0.8	0.4	1.1
3	salicylate	1.4	0.5	1.3
4	piroxicam	0.6	0.3	1.0
5	tiaprofenic acid	1.2	0.3	1.5

* Scored on a scale from 0-2.
• Scored on a scale from 0-4; 4 meaning complete depletion.
Values represent the mean calculated from groups of 12 mice.

tion also tended to be higher pointing to more preceeding inflammation. The following experiment was set up to repeat this observation and to see whether the potential stress of daily oral gavage can be avoided by putting the drugs in the drinking water. Prednisolone was included as a positive control. Table 3 shows the Tc-uptake values in animals treated with drug waters. In one experiment Tc-uptake was only measured at day 4, in a second experiment measurements were done at days 4, 7 and 14 and animals were sacrificed at day 28 for histology.

Table 3. Effect of drugs in drinking water on arthritis.

		R/L ratio of ^{99m}Tc-uptake#			
Group	Treatment	Day 4 •	Day 4	Day 7	Day 14
1	oral gavage$^{\Delta}$	1.97±0.32	1.73±0.21	1.33±0.17	1.16±0.07
2	water	1.86±0.16	1.55±0.25	1.23±0.12	1.14±0.10
3	salicylate	2.07±0.42	1.39±0.18	1.30±0.11	1.16±0.08
4	piroxicam	1.41±0.12*	1.27±0.07*	1.16±0.11	1.13±0.06
5	tiaprofenic acid	1.69±0.37	1.51±0.29	1.29±0.12	1.18±0.09
6	prednisolone	1.45±0.20*	1.48±0.24	1.16±0.14	1.11±0.10

Mean±SD calculated from groups of at least 9 mice.
• Day 4 measured in a separate experiment.
Δ Twice a day 250 µl tap water/mouse per os.
* Statistically significant difference compared to group 2.

The results clearly indicate that stress induced by daily oral gavage does not necessarily cause suppression of joint inflammation (comparison of groups 1 and 2). The Tc-uptake measurements in group 1 were even higher, although not reaching statistical significance. Furthermore, piroxicam (2 times) and prednisolone (once) showed significant suppression at day 4 (Table 3). At day seven slightly decreased values were still noted for these two drugs, not reaching statistical significance anymore.

To become informed about the average drug intake, the water consumption was measured daily. Average values over the whole 4 week period are shown in Table 4. The daily doses (ml) of group 1 (inclusive 500 µl by oral gavage), tiaprofenic acid and prednisolone group approximated the amounts of water intake by control mice (group 2). However, the salicylate and piroxicam treated mice drunk significantly less than those of group 2. Undoubtedly, the taste of these drug waters will be the reason for this. The average weight of the mice at the beginning (day -1) and at the end (day 28) of the experiment are also given in Table 4. Only prednisolone treatment appeared to result in significant weight loss.

Histological grading of the inflammation and cartilage damage is

Table 4. Average drug dose and water intake during, and weight change after, a 4 week period.#

Group	Treatment	Conc. mg/ml	Water intake ml/mouse/24h	Dose mg/kg/24h	Mouse weight(mg): day-1	day 28
1	oral gavage	-	2.6±0.5	-	26±2	26±2
2	water	-	3.3±0.4	-	27±2	29±2
3	salicylate	3	2.2±0.4*	255	26±2	28±2
4	piroxicam	0.2	2.7±0.4*	20	28±2	28±2
5	tiaprofenic acid	0.2	3.3±0.3	25	27±2	28±2
6	prednisolone	0.02	3.2±0.3	2.5	28±2	25±2 •

Values depict mean(±SD) obtained from at least 9 mice.
* Significant decrease in water intake, $p<0.01$.
• Significant decrease in weight, $p<0.05$.

shown in Table 5. Of interest, neither drug caused a significant aggravation of the cartilage damage. Antiinflammatory effects in terms of decreased values for infiltrate and exudate seem apparent for prednisolone and to a lesser extent for salicylate. Piroxicam seemed only to suppress exudation.

Apart from effects on cartilage proteoglycan depletion, we also looked for potential drug effects on chondrocyte integrity. Scores for chondrocyte death were similar for groups 1-5 and tended to be slightly lower for group 6 (data not shown).

Analysis of chondrocyte function. As mentioned above no abnormalities were found on gross observation of the articular cartilage of the left normal joints of mice treated for 4 weeks with the various drugs. To examine in more detail potential effects on chondrocyte synthetic function, additional groups of normal mice were treated with tiaprofenic acid and salicylate for 7.5 weeks. Twenty-four hours after the last drug treatment, mice received an intraperitoneal injection of $Na_2{}^{35}SO_4$ and 3 h later pateallae were isolated.

Anyway, no suppression of chondrocyte synthetic function was found in the drug treated groups (Table 6). In addition to quantitative analysis of chondrocyte function shown in Table 6, qualitative analysis was done by autoradiography. No evidence was obtained for any local

Table 5. Histological grading of arthritis.§

Group	Treatment*	Infiltrate in synovium	Exudate in joint space	Cartilage PG depletion
1	oral gavage	1.9	0.6	1.0
2	water	1.9	0.7	0.7
3	salicylate	1.2	0.2	0.5
4	piroxicam	1.7	0.2	1.3
5	tiaprofenic acid	1.6	1.0	1.0
6	prednisolone	0.7	0.0	0.4

§ See for histologic parameters Table 2.
* Drugs given in drinking water.

Table 6. Weight change of drug treated mice and ^{35}S-sulfate incorporation in articular knee cartilage.

Group	Drug	Dose • mg/kg	weight$^{\Delta}$ day 0	weight$^{\Delta}$ day 52	^{35}S incorporation$^{\#}$ patellae	^{35}S incorporation$^{\#}$ (%)
1	tiaprofenic acid	32	20±2	25±1	302±25	131*
2	"	16	20±2	25±1	275±29	119
3	"	8	20±2	24±2	252±27	109
4	"	4	19±2	25±1	349±48	151*
5	salicylate	200	19±2	25±1	338±50	146*
6	water	-	20±2	23±2	231±25	100

• Once a day by oral gavage.
Δ mg±SD of at least 12 mice.
\# cpm ± SD in the patellar cartilage of groups of 6 mice.
* $p<0.01$.

change in synthetic activity in the patellar cartilage, nor for the occurrence of dead chondrocytes.

Perhaps the increased synthetic activity found in some tiaprofenic acid groups and the salicylate group (Table 6) points to an attempt at repair after a period of suppressed synthetic activity. For instance, if drugs cause short-term suppression followed by a period of enhanced synthesis, the overall effect is probably negligible. Such reversibility was already demonstrated for the salicylate mediated suppression of ^{35}S-GAG synthesis after a single dose. Another aspect, apart from the amounts of GAG present in the cartilage, is the quality. If, in the phase of suppressed synthesis, molecules of a changed composition are made, e.g. undersulfated GAGs, this would disturb cartilage integrity. Methods have now been developed to address this issue shortly.

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CHAPTER 9

FINAL REMARKS AND DISCUSSION

FINAL REMARKS AND DISCUSSION

The study described in this thesis was aimed to elucidate iatrogenic effects, mediated by antirheumatic drugs, on (pathological) articular cartilage. Many conclusions from the present investigation, which has been centered around the cartilage of the mouse patella (kneecap) and the effects of nonsteroidal antiinflammatory drugs (NSAIDs) on its metabolism, are mentioned in Chapter 1. In that Chapter an overview is given of existing variations in drug test systems, in vitro as well as in vivo; some of these, undoubtedly, are the cause of variations in data obtained.

Of the NSAIDs examined, sodium salicylate was studied in detail and was furthermore used throughout the whole study as a reference drug. Before discussing the effects of several NSAIDs, some remarks are made on the cartilage test system, also used throughout the study.

The mouse patella assay. Since its development in 1982 at our laboratory (1), the mouse patella assay appeared very useful for studying chondrocyte metabolic activity in articular cartilage. An extension of this assay, merely confined to the metabolism of glycosaminoglycans (GAGs), is described in Chapter 2. It appeared that on the one hand ^{35}S-sulfate is a good radiolabeled precursor for quantitative studies on sulfation of GAGs. On the other hand, ^{3}H-glucosamine, but not ^{3}H-acetate or ^{3}H-glucose, is a suitable label for quantitating GAG backbone metabolism, both in vitro and in vivo. In our cartilage/drug studies this cartilage assay is employed which is a peculiar assay for two reasons:

1. Use is made of anatomically intact articular cartilage to perform in vitro incubation experiments; as distinct from frequently used cartilage slices, no artificial disrupted collagen meshwork, in which PGs and chondrocytes are entangled, is present. The direct environment of the chondrocytes, an intact extracellular matrix, is not changed compared to the in vivo situation.

2. Chondrocyte metabolic activity, defined as the amount of incorporated radiolabel per time unit (anabolic activity) or the amount lost from prelabeled patellae after a particular chase period (catabolic activity), is expressed per whole patella which is a well defined anatomical structure. In contrast with conventional assays, there is no need to

measure contents of cartilage constituents (e.g. DNA, uronic acid, hexosamine) or dry/wet weights, parameters against which incorporated radio amounts are usually expressed. This absence of an extra parameter determination makes the patella assay unique as an assay of mature articular cartilage and, probably, is the reason for the relative high accuracy of measurements (taken into account the interindividual differences between mice, the coefficient of variance is often below 20 %). A minor disadvantage might be that in this way only an overall metabolic activity is measured not accounting for differences in very local metabolic activities in the patellar cartilage layer. If particular chondrocytes or groups of chondrocytes would be more susceptible to drugs, autoradiography of whole sections of radiolabeled patellae (e.g. with $^{35}SO_4$) might be helpful in picking up such local differences. For example, after incubation with sodium salicylate, autoradiography of ^{35}S-labeled patellae revealed that at the marginal (synovial) regions of the patellar cartilage a high reduction of radioactivity was found, and this was not evident at the central part (Figure 1). Chondrocytes in the marginal regions are apparently more susceptible to salicylate than chondrocytes lying merely in the central part of the patellar cartilage. Of interest, if these synovial sides are regions which are habitually less loaded than the central cartilage part, then our observation is in accordance with that of Palmoski and Brandt. They found that habitually unloaded canine articular cartilage was susceptible to the suppressive action of salicylate, whereas loaded cartilage was not (2).

Other cartilage assays. Since the patella possesses no epiphyseal cartilage, its cartilage is entirely hyaline articular cartilage; following radiolabel incorporation the whole patella (freed from surrounding tissue) can be processed for measuring cartilage proteoglycan (GAG) metabolism (Figure 1). Searching for other suitable structures with anatomically intact articular cartilage we tried to use whole femoral heads. Besides a small interruption in the central part of the articular cartilage, caused by the attached ligamentum teres femoris, these femoral heads furthermore possess epiphyseal cartilage (Figure 2). Studying strictly articular cartilage metabolism, the epiphysis must be

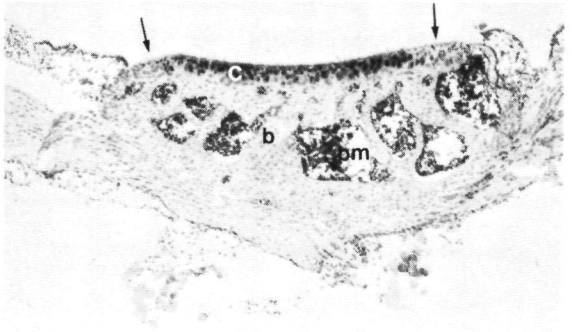

Figure 1. Autoradiograph of a section of a whole mouse patella, previously exposed to 10^{-2} M salicylate, embedded in the natural surrounding tissue and labeled with ^{35}S-sulfate. Note the reduction of black silver granules at the marginal regions of articular cartilage (arrows) and the absence of these granules in non-cartilaginous tissue; no epiphyseal cartilage is present. When used for quantification of the incorporated radiosulfate, the patella is punched out of the adjacent tissue. Stained with hematoxylin and eosin (H & E); original magnification 40x. c=articular cartilage; b=bone; bm=bone marrow.

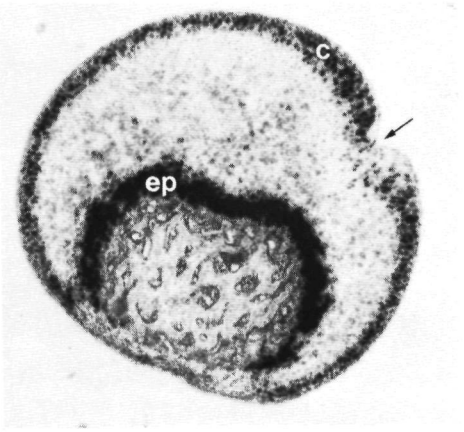

Figure 2. Autoradiograph of a section of a whole mouse femoral head, labeled with ^{35}S-sulfate. Radioactivity is seen above the articular cartilage layer (c) and, more heavily, above the epiphyseal cartilage (ep). The arrow points to the site at which the ligamentum teres femoris had been attached. Stained with H & E; original magnification 40x.

removed proceeding to radiolabel incorporation; till now we did not succeed in adequate separation of these two cartilage entities. Nevertheless, in vitro studies with sodium salicylate revealed that the overall synthetic activity (^{35}S incorporation rate) of femoral head chondrocytes (a mixture of both articular and epiphyseal ones) is influenced to the same extent as patellar chondrocytes, after short-term exposure to the drug (Table 1).

The patella is the largest sesamoid bone of the body and there are many more of these structures, which as a rule develop within tendons or ligaments (e.g. the fabella). It seems worthwile to examine these cartilage specimens for potential suitability in articular cartilage assays.

Susceptibility of patellar cartilage to salicylate in vitro. The potential effects of NSAIDs on the metabolism of articular cartilage of the mouse were started with salicylate. Salicylate is considered as the prototype of NSAIDs and is often used as a first choice drug in antirheumatic therapy. Studying the in vitro effects of therapeutic and higher drug concentrations on patellar cartilage, an inhibition of GAG synthesis was observed (see Figure 1 and Table 1); no disturbance was found on basal GAG degradation. This drug mediated suppression was not

Table 1. Salicylate effect on ^{35}S incorporation in whole patellae and in whole femoral heads: dose-response.*

Sodium salicylate (mol/l)	^{35}S content (counts per minute/specimen)#	
	Patella	Femoral head
0	919 ± 76 (100%)	2524 ± 491 (100%)
10^{-3}	855 ± 110 (93%)	2070 ± 465 (82%)
2×10^{-3}	689 ± 69 (75%)	2085 ± 316 (81%)
5×10^{-3}	568 ± 80 (62%)	1820 ± 486 (72%)

* Four h salicylate (without serum); final two h with ^{35}S-sulfate.
\# Results represent the mean ± SD of cartilage specimens from 6 mice.

only evident in patellar cartilage of young male C57Bl mice (up to 27 weeks; Chapter 5) but also in patellae of older mice (up to 1 year), female mice and mice of other strains (Swiss and "129"). Of interest, no change in magnitude of suppression was noticed, indicating that sex, age and strain differences do not effect mouse patellar cartilage's susceptibility to salicylate.

To obtain articular cartilage with higher susceptibility to salicylate, we also did some attempts to mimic the experiments performed by Palmoski and Brandt (3). They treated articular cartilage slices with hyaluronidase to deplete the matrix of proteoglycans (PGs). Subsequently, upon cultivation of these treated slices for several hours, GAG synthetic rate increased as did the chondrocyte susceptibility to salicylate (Chapter 1: Table 6). Decreased PG content, higher GAG synthetic rate and (much) higher susceptibility to salicylate was also demonstrated in osteoarthritic cartilage (4). However, with our mouse patella assay we did not succeed in demonstrating these phenomena. Hyaluronidase treatment did not affect anatomically intact patellar cartilage at all; trypsin treatment (Chapter 7) decreased PG content as well as the rate of GAG synthesis, but salicylate susceptibility was not enhanced. Augmented GAG synthesis was only exhibited by patellar cartilage in postarthritic mouse knee joints (day 28 after zymosan-induced arthritis; Chapter 7). Nevertheless, these affected patellae were not convincingly more susceptible to salicylate than normal patellae (in vitro); on the other hand there are no clear indications that PG content in these patellae would be decreased significantly. Perhaps the recently published glucose oxidase-induced arthritis model in mice, developed at our laboratory by Schalkwijk et al (5), is suitable for articular cartilage studies in the aforementioned sense. Already after one week the GAG synthetic rate in PG-depleted patellar cartilage is increased to about 200% (in vitro) and this condition is maintained at least for another 7 days (observations made by Drs. H. van Beuningen). Cartilage/drug assays with patellae from these arthritic joints is now under investigation.

An important point to make is that in our studies, described above, no use was made of serum in the medium in which the patella specimens were cultured; thus salicylate was essentially in the free form. In the presence of mouse serum, 25% or more, therapeutic concentrations of sa-

licylate exerted no effect on normal GAG metabolism anymore (Chapter 4 and 5). This was also found if albumin (2-4%) was added to the medium (Chapter 5), indicating that the remaining free (not protein bound) concentration of salicylate was not able to do any harm to patellar chondrocytes; low, free salicylate concentrations (10^{-5}-10^{-4} M) even tended to stimulate basal GAG synthesis (Chapter 5)! These latter observations indicate that a therapeutic dose of salicylate in vivo would have no direct suppressive effect on patellar chondrocytes in normal joints.

Susceptibility of patellar cartilage to salicylate in vivo. In Chapter 4 in vivo studies are described with surprising outcomes. Shortly after administration to mice, salicylate (200 mg/kg) induced a sulfate diuresis leading to a marked decrease in serum sulfate. Taken into account possible changes in specific radioactivity between drug treated animals and control ones, labeling studies with ^{35}S-sulfate and ^{3}H-glucosamine revealed that sulfate incorporation into GAGs of patellar cartilage was suppressed (Chapter 4), whereas glucosamine incorporation proceeded unaltered in vivo (6). The incorporation behaviour of these radiolabels in vivo suggests that salicylate treatment induced suppression of GAG sulfation without a concomitant disturbance in GAG chain elongation. Since salicylate in vivo presumably did not affect normal chondrocyte function directly (as stated above), the phenomenon of decreased sulfation had to occur via an indirect drug effect e.g. the observed, salicylate induced, decrease in availability of endogenous sulfate. This was studied in detail in vitro.

Exposing whole patellae to sulfate concentrations extending from physiological (mouse-)levels (about 1.0 mM SO_4) to almost nil, incorporation of sulfate in patellar GAGs was markedly reduced below a certain treshold concentration, i.e. 0.5 mM SO_4 (Chapter 4). Of interest, under these conditions no significant change in glucosamine incorporation was observed (Figure 3), suggesting undersulfation of GAGs. So far, these additional in vitro observations might explain the in vivo effects caused by salicylate in mice. As far as undersulfation is concerned, salicylate demonstrated another interesting aspect in vitro. At high drug concentrations, sulfate incorporation was more suppressed than glucosamine incorporation, suggesting again undersulfation of GAGs,

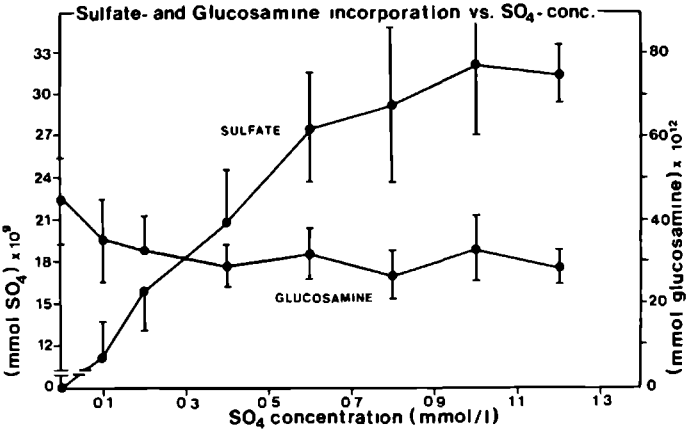

Figure 3. Sulfate and glucosamine incorporation into patellar glycosaminoglycans at various sulfate concentrations. Results are expressed as nanomoles of precursors incorporated per patella in 2 h and represent the mean ± SD of at least 5 mice.

(Chapter 5), now by a direct effect of salicylate on the chondrocyte. This observation could be related to the observation made by others that salicylate, under certain conditions, strongly inhibits sulfate transport across cell membranes (7).

Noteworthy, in this respect, is that incubation experiments performed with sulfate concentrations below 0.5 mM (without serum) augmented the degree of salicylate provoked suppression of sulfated GAG synthesis (Chapter 5).

Undersulfation of GAGs. Because of these, from a fundamental point of view, interesting observations we examined if low sulfate availability indeed could induce undersulfation of GAGs as suggested by Figure 3. To this end ^{3}H and ^{35}S labeled GAGs, isolated from patellar cartilage, were analysed with cellulose acetate (CA) electrophoresis. No clear difference in migration pattern of radiolabeled GAGs could be demonstrated as would be expected if undersulfated GAGs, possessing a lower negative charge density than normal GAGs, were present. Also, preliminary analysis (performed at the Jan van Breemen Institute, Amsterdam) with high performance liquid chromatography (HPLC) of sulfated disac-

charides, obtained after chondroitinase ABC digestion of radiolabeled GAGs, did not result in a clear cut answer as to undersulfation or not. Experiments done by Sobue (8) and Ito (9) on chick embryo cartilage, clearly demonstrated (by means of CA electrophoresis and ion exchange chromatography) that low environmental sulfate in vitro caused undersulfation of GAGs. This was also observed very recently by Humphries et al (10), studying bovine aortic endothelial cells, and Tyree et al (11), studying tumor cells. We do not know yet why we could not find any change in GAG properties, but this interesting matter is still under investigation. As a final remark to this issue we like to mention that also plants demonstrate a sulfate dependence like that described here (12).

Aspirin, the prototype of NSAIDs, is an interesting drug with respect to our studies in mice. Although we have not studied this particular drug in vivo yet, after absorption in the body it is fastly hydrolysed to salicylate, a drug which causes a sulfaturic effect leading to a decreased endogenous sulfate availability (Chapter 4). Nowadays newly developed derivatives of salicylate are on the market (e.g. diflunisal, benorylate, eterylate) and it would be of interest to study their actions on sulfate homeostasis in the mouse.

Sulfate determination. Because a suboptimal availability of inorganic sulfate in the circulation may suppress sulfation of GAGs and even might, on a chronic basis, deplete articular cartilage of (ester)sulfate, it is important to have the disposal of a sensitive sulfate assay, to be able to assess potential changes in sulfate levels in small experimental animals. Moreover, using ^{35}S-sulfate in vivo it is of utmost importance to be informed about the specific activity (ratio of $^{35}SO_4$ over endogenous SO_4) of this radiolabel in the circulation throughout the course of the experiment. For this purpose a simple scaled-down inorganic sulfate assay, derived from an existing sensitive benzidine method was developed (Chapter 3). This assay will substantially contribute to a reduction in the number of small animals, used for studies on inorganic sulfate. Amounts in the range of 1-12 nmol sulfate can easily be measured; for that, only 10 µl serum or cartilage from one mouse patella is needed.

Susceptibility of patellar cartilage to other NSAIDs: short-term and long-term drug exposure: Short-term in vitro and in vivo studies on the effects of tiaprofenic acid, a new developed NSAID, on normal patellar cartilage are described in Chapter 6. In addition the influence of chronic administration (7.5 weeks) of salicylate and tiaprofenic acid on the integrity of articular cartilage in normal mice is briefly discussed. Tiaprofenic acid exerted no effect on patellar GAG metabolism in vitro; in vivo this drug demonstrated a slight, but not significant, suppression of articular GAG synthesis. Chronic administration of tiaprofenic acid (and salicylate) to normal mice led to no change in histological features of knee joint cartilage.

Short-term effects of salicylate, tiaprofenic acid and piroxicam were further subjected to a detailed investigation in vitro and in vivo on healthy as well as arthritic cartilage. The steroid, prednisolone, was included in these studies as a highly effective antiinflammatory, but potentially harmful agent for articular cartilage. Proteoglycan depleted cartilage, obtained after trypsin treatment or taken from inflamed arthritic joints, was not convincingly more susceptible to these drugs, in vitro, than normal cartilage; except for salicylate these drugs seemed harmless (Chapter 7). In vivo effects were tested in mice with unilateral, zymosan-induced arthritis. Except for salicylate, the drugs did not affect endogenous serum sulfate levels to a significant extent, and in vivo patellar GAG synthesis (quantitated with ^{35}S-sulfate) was not influenced, neither in healthy, nor in arthritic patella. On the other hand, drug studies performed in mice with 28 day-lasting monoarthritis, were of particular interest. The affected joints, exhibiting augmented patellar GAG synthesis, were susceptible to suppressive actions of the NSAIDs. Again GAG synthesis in patellae of the normal, contralateral joints was not affected by tiaprofenic acid and piroxicam. In addition, studies were performed on GAG degradation in vivo. Under certain conditions only tiaprofenic acid and to a lesser extent prednisolone were able to suppress both the basal and enhanced (inflammation mediated) breakdown rate. These latter observations indicate that effects of drugs are dependent on the condition of cartilage and should stimulate future research on this item. In conclusion, as far as in vivo patellar GAG metabolism is concerned, only salicylate seems potentially detrimental, piroxicam seems neutral in action and

tiaprofenic acid and prednisolone seem to possess beneficial properties.

To extend these observations, above findings were put to the proof by performing long-term studies in mice with antigen induced arthritis (Chapter 8). Although prednisolone, piroxicam and salicylate, but not tiaprofenic acid, exerted some antiinflammatory effects, daily drug treatment, for 4-7.5 weeks, did not result in clear detectable changes, neither in normal knee-joint cartilage nor in damaged, arthritic cartilage. These latter results are still preliminar, and await completion by data from further investigations in this matter.

All the studies described in this thesis were performed to examine the potential role of NSAIDs in the process of articular cartilage destruction, occuring in chronic joint diseases. No indications were obtained for any marked deleterious effect, nor for the reverse, amelioration of damaged, arthritic cartilage by the studied NSAIDs. Because our studies were performed on mice, the relevance of outcomes should be further investigated, preferably in animals which possess more human-like (patho)physiology, before a definite conclusion can be made regarding iatrogenic cartilage effects of the drugs considered in this thesis.

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CHAPTER 10

SAMENVATTING

KRAAKBEEN VAN DE MUIZEPATELLA EN DE GEVOELIGHEID HIERVAN VOOR NIET-STEROIDALE ONTSTEKINGSREMMENDE MIDDELEN

Het promotieonderzoek, beschreven in deze dissertatie, heeft zich voornamelijk geconcentreerd op het kraakbeen van de muizepatella (knieschijf) en de beinvloeding van metabolische processen hierin door niet-steroidale ontstekingsremmende middelen (NSAIDs). Vele conclusies zijn reeds vermeld in hoofdstuk (HS) 1, waarin een overzicht wordt gegeven van bestaande variaties in geneesmiddeltestsystemen, zowel in vitro als in vivo, die ongetwijfeld voor een deel verantwoordelijk zijn voor de variaties in gegevens afkomstig van diverse onderzoekingen.

Een uitbreiding van de muizepatella-testmethode, ontwikkeld op ons laboratorium in 1982, wordt beschreven in HS 2. De patella wordt gebruikt als een bron van anatomisch intact gewrichtskraakbeen en blijkt uitermate geschikt voor de bestudering van effecten van ontstekingsmediatoren en NSAIDs op de stofwisseling van proteoglycanen (PGs), reuze molekulen die naast collageen en water de hoofdbestanddelen van kraakbeen vormen. Inbouw van ^{35}S-sulfaat, dat gewoonlijk wordt gebruikt om de synthesesnelheid van de hoog gesulfateerde glycosaminoglycanen (GAGs), de belangrijkste componenten van PGs, te meten, werd nauwkeurig bestudeerd. Ook andere radiolabels (^{3}H-glucosamine, ^{3}H-glucose, ^{3}H-acetaat), in gebruik voor de bestudering van de GAG-ketenverlenging, werden op hun geschiktheid onderzocht.

De ontwikkeling van een eenvoudige analysemethode voor zeer kleine hoeveelheden anorganisch sulfaat, afgeleid van een bestaande gevoelige benzidinemethode en beschreven in HS 3, zal aanzienlijk minder kleine proefdieren vergen, welke worden gebruikt voor studies gericht op het sulfaat-metabolisme. Hoeveelheden van 1-12 nmol sulfaat kunnen makkelijk gemeten worden; daarvoor is slechts 10 µl serum of kraakbeen van één muizepatella nodig.

Studies naar de effecten van NSAIDs op het metabolisme van normaal gewrichtskraakbeen van de muis werden begonnen met salicylaat in een therapeutische, ontstekingsremmende dosis. Salicylaat wordt wel beschouwd als het prototype van NSAIDs en wordt vaak (niet in Nederland) als geneesmiddel van eerste keus gebruikt in de medicamenteuze therapie van reumapatienten. In HS 4 worden in vivo studies beschreven met verrassende resultaten: 1. Salicylaat veroorzaakt een sulfaatdiurese die

een sterke daling van de endogene, anorganische sulfaat-spiegel tot gevolg heeft. 2. De verlaagde sulfaat beschikbaarheid, op zich, werd verantwoordelijk gesteld voor de waargenomen onderdrukking van de gesulfateerde GAG-synthese in de muizepatella. Om de in vivo salicylaat effecten beter te kunnen verklaren werden de in vitro effecten van salicylaat op het patellaire kraakbeen GAG-metabolisme onderworpen aan een nader onderzoek (HS 5). De suppressieve eigenschappen van therapeutische concentraties salicylaat verdwenen in de aanwezigheid van 25% of meer serum in het incubatiemedium, hetgeen doet vermoeden dat salicylaat in vivo geen direct effect uitoefent op het metabolisme van de kraakbeencel (chondrocyt).

Kortdurende studies naar in vitro en in vivo effecten van salicylaat en tiaprofeenzuur, een nieuw ontwikkeld NSAID, op kraakbeen van gezonde muizen worden beschreven in HS 6. Ook wordt de invloed van chronische toediening (7,5 week) van deze medicamenten aan normale muizen kort besproken. Geen indicatie werd verkregen voor enig schadelijk effect op kniegewrichtskraakbeen na deze (relatief) langdurige behandeling.

Studies naar de effecten van NSAIDs op pathologisch kraakbeen zijn natuurlijk relevanter dan op gezond kraakbeen. HS 7 behandelt kortdurende studies van in vitro en in vivo effecten van diverse NSAIDs, in het bijzonder de nieuwe farmaca piroxicam en tiaprofeenzuur, op zowel gezond kraakbeen als kraakbeen van ontstoken muizeknietjes. Het steroïd prednisolon werd in deze studies gebruikt om te fungeren als een sterk ontstekingsremmend middel en tegelijkertijd als een mogelijk schadelijke stof voor gewrichtskraakbeen. Piroxicam leek neutraal in werking, terwijl tiaprofeenzuur en prednisolon zelfs een heilzame werking uitoefenden op kraakbeen.

Als een logische voortzetting werden de effecten van NSAIDs na chronische toediening (dagelijks, 4 weken lang) aan muizen met antigeen-geïnduceerde, eenzijdige gewrichtsontsteking, bestudeerd (HS 8). De resultaten zijn nog steeds voorlopig van karakter en het is op dit moment niet mogelijk om harde conclusies te trekken over heilzame of nadelige effecten van deze medicijnen op gewrichtskraakbeen van ontstoken knietjes. In ieder geval, opvallend schadelijke effecten werden nooit gevonden, noch voor normaal kraakbeen noch voor kraakbeen uit ontstoken gewrichten.

Ten slotte, naast het breken van een lans voor de patella-testmethode, wordt in HS 9 een overzicht gegeven van de behaalde resultaten. Verder worden additionele gegevens vermeld, in het bijzonder over relaties tussen salicylaat, anorganisch sulfaat en ondersulfatering van glycosaminoglycanen.

Zoals uit de namen van de auteurs en de dankbetuigingen, vermeld in vele hoofdstukken, moge blijken, is het werk dat in dit proefschrift staat beschreven tot stand gekomen met de steun en de hulp van diverse mensen. Ik dank hen allen. Speciaal Elly Vitters wil ik bedanken voor haar grote aandeel in het praktisch werk, in het bijzonder voor de bereidheid vele sulfaatbepalingen, die soms tot wanhoop leidden, weer opnieuw op hun deugdelijkheid te testen. De medewerkers van het Centraal Dierenlaboratorium, met name GJ Grutters en H Eikholt, ben ik erkentelijk voor het verzorgen van de muizen en voor de bijdrage in het dierexperimenteel onderzoek ten tijde dat toediening van medicijnen aan de orde van iedere dag was. Voor het dagenlange werk van het drukklaar maken van de manuscripten ben ik dank verschuldigd aan Marion Janssen die op zwakke momenten met vaardige hand de scepter zwaaide; samen hebben we de tekstverwerker nog beter leren beheersen. Voorts ben ik dankbaar voor de prettige samenwerking met medewerkers van de afdeling Reumatische Ziekten, voor de goede werksfeer en voor de genoten wetenschappelijke vrijheid.

CURRICULUM VITAE

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Geboren op 24 augustus 1951 te Brunssum.

1968 Diploma MULO-B; Koningin Juliana School te Treebeek.

1972 Diploma HBO-B, Analytische Chemie; Zuidlimburgse Laboratoriumschool te Sittard.

Katholieke Universiteit Nijmegen

1978 Examen deskundigheid stralingshygiëne voor een C-laboratorium.

1980 Doctoraalexamen Scheikunde (S2).
Hoofdvak: Biochemie.
Bijvakken: Exobiologie en Algemeen Gedeelte (Quantumchemie, Instrumentele Methoden II, Chemische Technologie, Ethiek).

1980 Onderwijsbevoegdheid in Scheikunde.

Werkzaamheden aan het Sint Radboudziekenhuis te Nijmegen

1980 Immunologisch onderzoek op de afdeling Pathologische Anatomie (hoofd: Prof. Dr GP Vooijs) onder leiding van Dr RMW de Waal.

1982 Promotieonderzoek op het research laboratorium van de afdeling Reumatische Ziekten (hoofd: Prof. Dr LBA van de Putte) onder leiding van Dr WB van den Berg. Subsidies: Nederlandse Vereniging voor Reumabestrijding en (gedeeltelijk) Roussel BV en Pfizer International.

1986 Vervolg onderzoek, als wetenschappelijk medewerker, op het laboratorium voor Reumatische Ziekten, financieel gesteund door subsidie van de Universitaire Onderzoekspool.

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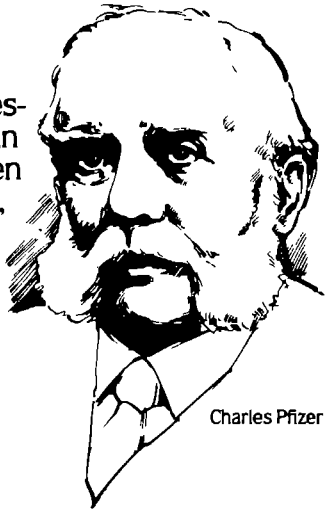

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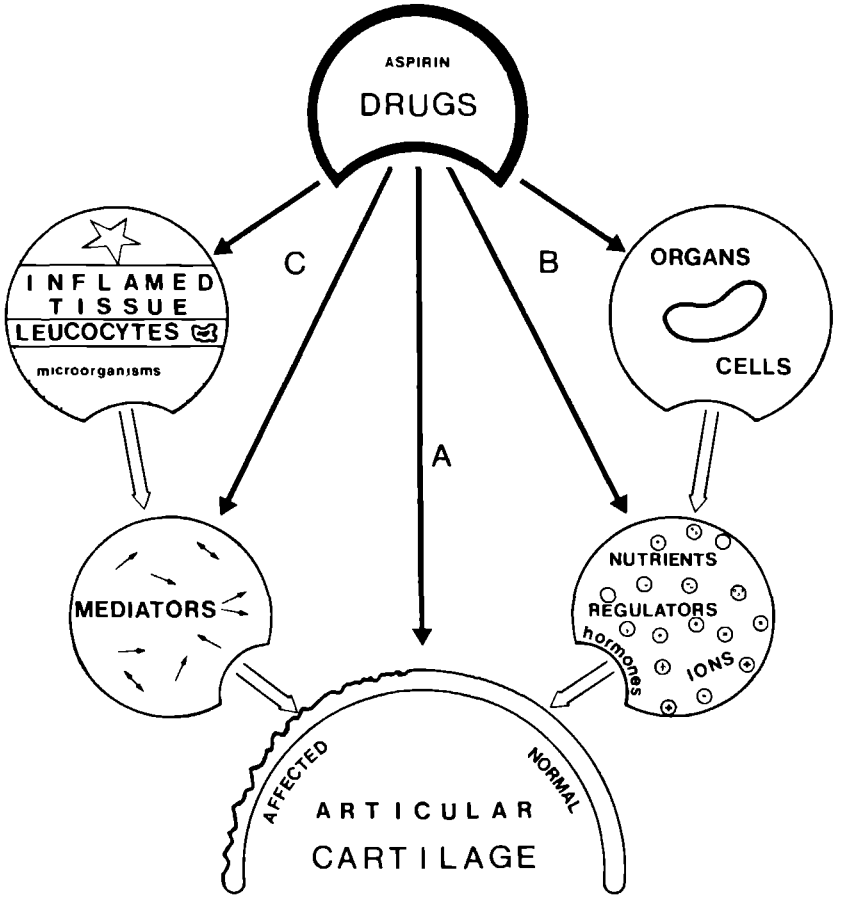

Bernard J. de Vries

STELLINGEN

1. De vermeende schadelijkheid van NSAIDs voor gewrichtskraakbeen is geenszins voldoende bewezen.
(Dit proefschrift)

2. Radiolabeling van de novo gesynthetiseerde gesulfateerde glycosaminoglycanen door chondrocyten in cultuurmedium met carrier-free anorganisch ^{35}S-sulfaat kan leiden tot ondergesulfateerde produkten.
(Dit proefschrift)

3. Na toediening van anorganisch ^{35}S-sulfaat wordt vaak ten onrechte aangenomen dat het "gedrag" van anorganisch sulfaat in vivo weerspiegeld wordt door dat van ^{35}S.
(Dit proefschrift)

4. In vitro effecten van antireumatica op kraakbeen dienen in aanwezigheid van serum of synoviaalvocht te worden bestudeerd.
(Dit proefschrift)

5. Verergering van experimentele osteoarthritis na chronische toediening van aspirine zou mogelijk te wijten kunnen zijn aan anorganisch sulfaat gebrek.
(Dit proefschrift)

6. De niet-carcinogene stof 4-amino-4'-chloordifenyl (CAD) lijkt een goede vervanger voor het carcinogene reagens, benzidine, dat gebruikt wordt in sulfaatbepalingen.

7. Aspirine is een prodrug voor salicylaat.
JR Vane et al, 2nd World Conference on Inflammation, Monte-Carlo, 1986.

8. Waarschijnlijk onderdrukken de niet-steroidale ontstekingsremmende pijnstillers (NSAIDs) over het algemeen meer de pijn dan de ontstekingsaktiviteit.

9. Het kniegewricht van de muis lijkt geschikt om de lokale bijwerkingen van het radiocolloïde Yttrium-90-silicaat, in gebruik voor bestralings-synovectomie, te bestuderen.
BJ de Vries et al, Agents Actions 15: 101-103, 1984.

10. Radioactief Yttrium penetreert mogelijk in het kraakbeen na intra-articulaire toediening van Yttrium-90-silicaat.

11. Het gebruik van het monoklonale anticytokeratine antilichaam CAM 5.2 in de tumordiagnostiek dient met gepaste voorzichtigheid te geschieden, aangezien dit reagens ook glad spierweefsel en spierweefseltumoren (leiomyosarcomas) herkend.
M Leader et al, Histopathology 10: 1315-1324, 1986.

12. Het is onbillijk dat de noodzakelijk geachte kortere verlenging van 't rijbewijs van invalide personen als consequentie heeft, dat telkens weer de normale onkosten moeten worden opgebracht.

13. Het verkrijgen van een pijnstiller uit de verpakking is voor een reumapatient soms een pijnlijke aangelegenheid.
Wouter Klootwijk, de Volkskrant (27-12-86).

14. De invoering van de term KLOIO's (klassiek opgeleiden in opleiding) voor diegenen die een klassieke akademische opleiding hebben genoten maar noodgedwongen op een AIO-plaats worden gestationeerd, dient te worden vermeden.

15. Het feit dat steeds meer medische publikaties verschijnen in boulevardbladen zal ertoe leiden dat ook voor deze "literatuur" een citatie-index wordt vastgesteld.

16. Na het doctoreren zullen vele doctorandi niet alleen een punt achter hun wetenschappelijke loopbaan zetten, maar ook achter de afkorting van hun nieuwe titel; hoewel dit laatste vaak onbewust gebeurt zijn hier geen duidelijke richtlijnen voor (zie van Dale, 11e druk 1984, onder de rubriek "Het gebruik van leestekens" en onder het woord "punt").

17. De dichte zijkant van een proefschrift blijft het langst in zicht.

18. Reclame in een proefschrift is iets dat nú nog opvalt.

19. Promoveren kost vriendschap.

BJ de Vries. Nijmegen, 20 februari 1987.